Measurement of ß-hydroxybutyrate in capillary blood obtained from an ear to detect hyperketonemia in dairy cows by using an electronic hand-held device

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zur Erlangung der Würde des
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DAVID SÜSS

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**Betreuer**

Dr. Michael Iwersen  
Universitätsklinik für Wiederkäuer, Abteilung Bestandsbetreuung für Wiederkäuer

**Gutachter**

Ao. Univ. Prof. Dr. Alois Strasser  
Department für Biomedizinische Wissenschaften  
Institut für Physiologie, Pathophysiologie und Biophysik
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1. Introduction

Ketosis in its subclinical (SCK) and clinical (CK) manifestation is a widespread metabolic disease in high producing dairy cows and occurs predominantly in the first weeks post partum. Analyzing the β-hydroxybutyrate (BHBA) concentration in serum or plasma is the reference test for detecting hyperketonemia (HYK), comprising CK and SK (Duffield et al. 1998). According to Oetzel (2004), CK is defined as ≥3.0 mmol/L BHBA and SCK is defined as a BHBA concentration of 1.2 to 2.9 mmol/L in blood. The average prevalence of SCK in 10 European countries within 2 to 15 days post partum (dpp) was 21.8%, using a threshold of ≥1.2 mmol of BHBA/L in whole blood (Suthar et al. 2013). For Western Europe, Berge and Vertenten (2014) presented a prevalence for HYK of 39% between d 7 and d 21 after calving based on a milk test for ketones. Mahrt et al. (2015) reported a mean prevalence of SCK within the first 42 dpp in Germany of 11.8%, using also a threshold of ≥1.2 mmol of BHBA/L. Blood from a tail vessel was tested with a hand-held device in this study.

A recent study from The Netherlands (Vanholder et al. 2015) reported major risk factors for SCK and CK in the second week of lactation: a moderate or high body condition score three to one week before the expected calving, an increased colostrum volume at first milking and an advanced parity. In Western European dairy herds, a smaller herd size, partially mixed rations instead of total mixed rations and also an advanced parity were associated with an increased risk for ketosis (Berge and Vertenten 2014). The duration of the risk period for HYK takes at least until 42 days after calving (Mahrt et al. 2015). A mean disease duration of 3 to 4 d days after calving (Mahrt et al. 2015) and a median duration of 5 d (McArt et al. 2012) have been reported recently. Current studies in Europe showed that elevated concentrations of BHBA in blood are associated with metritis, CK, displaced abomasum and lameness (Suthar et al. 2013, Berge and Vertenten 2014). Each 0.1 mmol/L BHBA increase at first positive test for SCK was associated with a decrease in milk production of 0.5 kg/d for the first 30 days in milk (DIM) (McArt et al. 2012). Furthermore, McArt et al. (2015) estimated the cost per case of HYK as approximately $289, reporting that main costs are caused by future losses in reproduction, milk production and culling or even through death loss.
Various electronic hand held devices have been evaluated for the measurement of BHBA in the recent years, and all tested devices were eligible as cowside test for the detection of SCK (Iwersen et al. 2009, Voyvoda and Erdogan 2010, Iwersen et al 2013, Mahrt et al. 2014b, Pineda and Cardoso 2015). Furthermore, several tests have been evaluated for dairy cows to detect ketones in milk (Geishauser et al. 1998, Geishauser et al. 2000, Carrier et al. 2004, Krogh et al. 2011) or urine (Carrier et al. 2004, Krogh et al. 2011). Mahrt et al. (2014a) evaluated three different sampling locations for BHBA determination. They concluded that blood drawn from the jugular vein or tail vessels should be used rather than samples obtained from the mammary vein, because of systematically lowered BHBA concentrations. Kanz et al. (2015) tested the suitability of capillary blood obtained from the skin of the external vulva for BHBA measurements using different hand-held devices in dairy cows. The authors reported that this technique is eligible to detect SCK. To our knowledge, the determination of BHBA concentration in capillary blood obtained from an ear using a minimally invasive sampling technique has not been described in cattle, yet. Potential benefits of this procedure could be the easier accessibility than the measurement at the external vulva, particularly when animals are fixed in headlocks for routine health checks, or if the vulva of the cows is very dirty.

The primary objective of the present study was to test whether capillary blood obtained by puncturing the skin of an ear with a minimal invasive lancet is eligible to detect HYK in dairy cows. Additionally, test characteristics of a new available hand-held device (FSP-Neo) for the determination of BHBA concentrations in bovine blood were evaluated.
2. Materials and Methods

2.1 Study design

The study was approved by the institutional ethics committee of the University of Veterinary Medicine (ETK-10/05/2015), Vienna, as well as by the Slovakian Regional Veterinary Food Administration.

The study was conducted in August 2015 on a commercial dairy farm in Slovakia, housing approximately 2,500 Holstein-Friesian cows. Animals were kept on deep straw bedding in a free-stall barn. The average energy corrected milk yield (based on 4.0% butterfat and 3.4% protein) was 9,165 kg in 2014. Study animals were multiparous cows from 1 dpp up to 21 dpp. To achieve a maximum irrelevant difference in the BHBA concentration of 0.1 mmol/L between the laboratory and capillary blood results with the hand-held device, a sample size calculation (type I error α=0.05, type II error β=0.2) was performed, resulting in 216 animals required. To compensate for possible data loss due to study animal exclusions (e.g. because of (pre-)analytical problems) 240 animals were sampled and used in this study.

An electronic hand-held device [FreeStyle Precision Neo (FSP-Neo, Abbott GmbH & Co. KG, Wiesbaden, Germany)] was used to analyze the BHBA concentration in capillary and coccygeal blood. To gain capillary blood, a disposable safety lancet [MiniCollect Safety Lancets, Greiner Bio-One International AG, Kremsmünster, Austria] was used, with a penetration depth of 2 mm and a blade width of 1.5 mm. Capillary blood samples were taken at three different locations: at the edge of the left and right ear and again on one of the two ears. The repeated measurement (2nd test) was performed alternately on one of the two ears and regarded as a third sampling location for capillary blood. The sampling procedures started with puncturing the skin of the left or right ear. The amount of blood necessary for BHBA measurements with the FSP-Neo was 1.5 µl. If the blood volume was insufficient for the measurement, the ear was punctured once again, approximately 1 cm lateral from the first puncture. If the obtained blood volume was still insufficient for a reliable measurement, the capillary bleeding was enforced by softly squeezing the skin of the ear. After inserting the test
strips into the hand-held device, the front edge of the strips was dipped directly onto the drop of blood. Additionally, blood samples from a coccygeal vessel from each cow were obtained with vacuum tubes coated with a clot activator for serum collection (Vacuette, 9 ml, Greiner Bio-One GmbH, Kremsmünster, Austria). Immediately after sampling, the BHBA concentration in whole blood was determined by dipping the sensor of the test strip from the FSP-Neo device onto the surface of the blood-filled serum tube. The coccygeal blood samples were analyzed with the FSP-Neo device to compare the test results for capillary and coccygeal blood and to describe test characteristics of the hand-held meter. Therefore the BHBA concentration of the coccygeal blood sample were determined in serum at the laboratory and used as reference.

After clotting at approximately 15°C for 2 h, the reference sample was centrifuged [(Eppendorf Centrifuge 5804, Eppendorf AG, Hamburg, Germany), 10 min, 18°C, 2,200 x g] to harvest the serum. The supernatant was divided in two aliquots and stored at a temperature of -18°C until further analyses at the laboratory of the Central Clinical Pathology Unit (CCPU), University of Veterinary Medicine, Vienna, Austria. Laboratory proceedings for the determination of the BHBA concentration at the CCPU were previously described by Pichler et al. (2014). The BHBA concentrations analyzed in serum at the CCPU were used as reference value in the present study.

Furthermore, 20 aliquot blood samples taken from the same cow were randomly placed between the samples obtained from the 240 study animals. These ‘standard samples’ were taken to evaluate the intra-assay variability of the laboratory analyses. Additionally, the intra- and interassay coefficients of variations (CV) were calculated for the FSP-Neo device in capillary blood. To evaluate the variability of the hand-held device measurements in capillary blood, three cows sampled with laboratory BHBA concentrations varying between 0.36 mmol/L to 1.86 mmol/L were used. Each animal was tested five times with the same device (intra-assay variability) and five times with different devices of the same type (inter-assay variability). The date of sampling, the animals identification number, the number of required punctures, squeezing and the measured BHBA concentrations of the hand-held device in capillary blood and coccygeal blood were recorded onto a pre-assigned data sheet.
2.2 Statistical analyses

SPSS Statistics for Windows (version 20.0; IBM Deutschland GmbH, Ehningen, Germany), Excel for Windows (version 14.0, Microsoft Corp., Redmond, WA), BiAS for Windows (version 10.06; Epsilon-Verlag, Darmstadt, Germany) and MedCalc for Windows (version 12.4; MedCalc Software, Ostend Belgium) were used for statistical analyses. For all statistical tests the level of significance was set at $P = 0.05$.

Data were tested for normal distribution by Kolmogorov-Smirnov test and nonparametric testing was initially performed. To describe the statistical association between variables, Spearman’s correlation coefficients ($\rho_s$) were calculated for the BHBA concentrations analyzed with the hand-held device thrice in capillary blood of the ear (i.e. left, right, repeated measurement) and in coccygeal blood, and with the reference method in the laboratory. The agreement between BHBA concentrations determined with the hand-held device and the values determined at the CCPU was evaluated by using the method described by Bland and Altman (1986).

According to the concentration of BHBA determined in serum at the CCPU, samples were classified as non-ketotic (<1.20 mmol/L) or subclinically ketotic ($\geq$ 1.20 mmol/L). Based on this classification, receiver operating characteristics (ROC) analyses were performed to calculate optimal thresholds for all four sampling locations (i.e. left, right ear, repeated measurement, coccygeal vessel). With these optimized thresholds, sensitivities (Se), specificities (Sp) and the Youden index (YI) were calculated. The area under the ROC curve (AUC-ROC) shows how well an estimated threshold can differentiate between two diagnostic groups (non-ketotic, subclinically ketotic) and represents the quality of this parameter according to Se and Sp (Swets 1988).

The variance between the BHBA concentrations determined with the FSP-Neo and the reference test was calculated. Moreover, mixed model analyses for repeated measurements were performed to evaluate if the sampling location, squeezing (classified in two different groups, until amount of blood was sufficient a ‘non squeezing group’ and if the amount was
insufficient a ‘squeezing group’), week of lactation or parity (2nd lactation or ≥ 3rd lactation) affected the BHBA concentration.
3. Results

3.1 Descriptives

In total, samples were taken from 240 dairy cows that where on average in the third lactation (min. = 2, max. = 7, IQR = 1) with a median of 7 dpp (min. = 0, max. = 21, IQR = 8). A total number of 25 cows exceeded a threshold of 1.2 mmol/L BHBA in serum, resulting in a prevalence of SCK of 10.4%. The median and mean ± SD BHBA concentration determined at the CCPU from all samples were 0.63 mmol/L (min. = 0.3 mmol/L, max. = 4.7 mmol/L, IQR = 0.2 mmol/L) and 0.76 mmol/L ± 0.57 mmol/L, respectively. Laboratory results and further descriptive statistical parameters for the BHBA concentrations measured in capillary and coccygeal blood using the FSP-Neo device are presented in Table 1.

Capillary blood from the ear could be obtained in 88.5% with the first puncture and in 98.8% with the first or second puncture. All together, 720 capillary blood samples and 240 samples from the coccygeal vessel were tested with the FSP-Neo hand-held device.

Table 1: Descriptive statistics of the BHBA concentrations measured in capillary and coccygeal blood using the hand-held device as well as in serum analyzed at the laboratory

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Serum²</th>
<th>FSP-Neo¹</th>
<th>Coccygeal blood</th>
<th>Capillary blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Laboratory</td>
<td>tail</td>
<td>left ear</td>
<td>right ear</td>
</tr>
<tr>
<td>Number of samples (n)</td>
<td>240</td>
<td>240</td>
<td>240</td>
<td>240</td>
</tr>
<tr>
<td>Mean (mmol/L)</td>
<td>0.76</td>
<td>0.79</td>
<td>0.98</td>
<td>0.99</td>
</tr>
<tr>
<td>SD (mmol/L)</td>
<td>0.57</td>
<td>0.66</td>
<td>0.73</td>
<td>0.72</td>
</tr>
<tr>
<td>Median (mmol/L)</td>
<td>0.63</td>
<td>0.60</td>
<td>0.80</td>
<td>0.80</td>
</tr>
<tr>
<td>Interquartile range (mmol/L)</td>
<td>0.20</td>
<td>0.30</td>
<td>0.50</td>
<td>0.40</td>
</tr>
</tbody>
</table>

¹ FSP-Neo: FreeStyle Precision Neo (Abbott GmbH & Co. KG, Wiesbaden, Germany)
² Obtained from coccygeal blood (reference test)
³ Laboratory of the Central Clinical Pathology Unit (CCPU), University of Veterinary Medicine, Vienna, Austria
⁴ 2nd test: Second puncture at the ear, approximately 1 cm lateral from the first one at the left or right ear
Measurement values from the three sampling locations for capillary blood showed a greater BHBA concentration compared with the reference test. The comparison of the results obtained from the hand-held meter for coccygeal and capillary blood against the reference test results are shown in Figure 1.

Figure 1. Differences of BHBA concentrations (mmol/L) obtained with the hand-held device at the four different sampling locations against results from the reference test.
3.2 Spearman correlation coefficient and Bland and Altman plots

The comparison of BHBA concentrations measured with the FSP-Neo at three different capillary blood sampling locations with the reference resulted in a Spearman correlation coefficient $\rho_s$ of 0.78, 0.76, and 0.81 for the left ear, the right ear and the repeated measurement, respectively (Figure 2, $P < 0.01$, for each parameter). Comparing the results of capillary blood from the different sampling sites yielded in $\rho_s = 0.77$ between the left and right ear and in $\rho_s = 0.81$ between the repeated measurement and the left and right ear ($P < 0.01$). The Spearman correlation coefficient $\rho_s$ for the BHBA concentration in the coccygeal blood measured with the hand-held device and the reference test was 0.92 ($P < 0.01$).

![Graph showing BHBA concentrations in mmol/L from the reference test against values obtained with the hand-held device at three different capillary sampling sites at the ears.](image-url)

**Figure 2.** BHBA concentrations (mmol/L) from the reference test against values obtained with the hand-held device at the three different capillary sampling sites at the ears.
The Bland and Altman plots in Figure 3a and 3b depict the differences between the values measured with the hand-held device and the reference test. Using capillary blood, the mean ± SD BHBA deviation against the reference test were 0.20 ± 0.47 mmol/L for all three capillary sampling locations, 0.22 ± 0.49 for the left ear, 0.23 ± 0.51 for the right ear, and 0.17 ± 0.41 mmol/L for the second test on the ear (positive bias, \(P < 0.01\)). Using coccygeal blood, the mean ± SD BHBA difference compared with the reference test was 0.02 ± 0.21 mmol/L (positive bias, \(P < 0.01\)).

**Figure 3a.** Bland-Altmann Plot of differences between BHBA concentrations obtained at the laboratory in serum and measured in capillary blood at three different capillary sampling locations with the FSP-Neo. The upper and lower solid lines illustrate the mean ± 2 SD, the solid line in the middle shows the mean.
Figure 3b. Bland-Altmann Plot of differences between BHBA concentrations obtained at the laboratory in serum and measured in coccygeal blood with the FSP-Neo. The upper and lower solid lines illustrate the mean ± 2 SD, the solid line in the middle shows the mean.
3.3 Mixed model analyses

The results of the first mixed model analyse on the influencing factors on the analyzed BHBA concentrations are presented in Table 2. Dairy cows in third or higher lactation showed significantly greater BHBA concentrations compared with cows in second lactation (no first lactating cows were tested in this study). Furthermore, cows sampled in the first week of lactation showed greater BHBA concentrations compared to cows in the third week of lactation. Sampling site showed no significant influence on the measured BHBA concentrations. However, significant effects of sample origin (capillary blood compared with reference test) on BHBA concentrations were found.

The second mixed model analyse was performed to evaluate, whether sampling site and squeezing influenced the difference in BHBA concentration between test results obtained from capillary blood at the ear and coccygeal serum analyzed at the CCPU (Table 3). Similar to the results of the first model, sampling site did not affect the difference between BHBA concentrations analyzed with the hand-held device and the reference after significance correction by Bonferroni (Bland and Altman 1995). Squeezing the sampling sites at the ears showed a significant difference in the mean BHBA concentrations against the reference test results. The difference in BHBA between the two groups (no squeezing/squeezing) was 0.06 mmol/L.
Table 2: Mixed model analyses on the influence of lactation class, lactation week and sampling site on measured BHBA concentrations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Comparison parameter</th>
<th>Mean¹ (Cl₉₅)³</th>
<th>Standard error r</th>
<th>Difference of mean² (Cl₉₅)³</th>
<th>Standard error r</th>
<th>Significance⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactation class</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Lactation ≥ 3. Lactation</td>
<td>≥ 3. Lactation</td>
<td>0.76 (0.70 to 0.81)</td>
<td>0.03</td>
<td>-0.18 (-0.26 to 0.11)</td>
<td>0.04</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>2. Lactation ≥ 3. Lactation</td>
<td>≥ 3. Lactation</td>
<td>0.94 (0.88 to 1.00)</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactation week</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Week 2. Week</td>
<td>3. Week</td>
<td>0.95 (0.90 to 1.00)</td>
<td>0.03</td>
<td>0.08 (-0.02 to 0.18)</td>
<td>0.04</td>
<td>0.16</td>
</tr>
<tr>
<td>1. Week 2. Week</td>
<td>3. Week</td>
<td>0.22 (0.08 to 0.35)</td>
<td>0.06</td>
<td></td>
<td></td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>2. Week 3. Week</td>
<td>3. Week</td>
<td>0.87 (0.80 to 0.93)</td>
<td>0.03</td>
<td>0.13 (-0.01 to 0.28)</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td>3. Week</td>
<td></td>
<td>0.73 (0.63 to 0.83)</td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling site</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left ear</td>
<td>Right ear</td>
<td>0.94 (0.85 to 1.03)</td>
<td>0.05</td>
<td>-0.01 (-0.19 to 0.18)</td>
<td>0.07</td>
<td>1.00</td>
</tr>
<tr>
<td>Left ear 2nd test</td>
<td></td>
<td>0.05 (0.13 to 0.23)</td>
<td>0.07</td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Left ear V. coccyea</td>
<td></td>
<td>0.19 (0.02 to -0.37)</td>
<td>0.06</td>
<td></td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>Left ear Serum</td>
<td></td>
<td>0.22 (0.05 to 0.39)</td>
<td>0.06</td>
<td></td>
<td></td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Right ear 2nd test</td>
<td></td>
<td>0.95 (0.85 to 1.04)</td>
<td>0.05</td>
<td>0.06 (-0.12 to 0.24)</td>
<td>0.07</td>
<td>1.00</td>
</tr>
<tr>
<td>Right ear V. coccyea</td>
<td></td>
<td>0.20 (0.03 to 0.38)</td>
<td>0.06</td>
<td></td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>Right ear Serum</td>
<td></td>
<td>0.23 (0.06 to 0.39)</td>
<td>0.06</td>
<td></td>
<td></td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>2nd test V. coccyea</td>
<td></td>
<td>0.89 (0.80 to 0.98)</td>
<td>0.05</td>
<td>0.14 (-0.03 to 0.32)</td>
<td>0.06</td>
<td>0.21</td>
</tr>
<tr>
<td>2nd test Serum</td>
<td></td>
<td>0.17 (0.01 to 0.33)</td>
<td>0.06</td>
<td></td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>V. coccyea Serum</td>
<td></td>
<td>0.75 (0.66 to 0.83)</td>
<td>0.04</td>
<td>0.03 (-0.13 to 0.18)</td>
<td>0.06</td>
<td>1.00</td>
</tr>
<tr>
<td>V. coccyea Serum</td>
<td></td>
<td>0.72 (0.65 to 0.79)</td>
<td>0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ BHBA concentration in mmol/L
² Difference of mean in mmol/L BHBA between parameter and comparison parameter
³ Cl₉₅: 95% confidence interval
⁴ Adjustment for multiple comparisons by Bonferroni
⁵ 2nd test: Second puncture at the ear, approximately 1 cm lateral from the first one at the left or right ear
⁶ Laboratory of the Central Clinical Pathology Unit (CCPU), University of Veterinary Medicine, Vienna, Austria
Table 3. Mixed model analyses to show the influence of squeezing and sampling site on the difference in BHBA concentrations between test results from capillary blood from the ear and coccygeal serum analyzed at the CCPU

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Comparison parameter</th>
<th>Difference of mean(^1) (CI(_{95}))(^3)</th>
<th>Standard error (r)</th>
<th>Difference parameter - comparison parameter(^2) (CI(_{95}))(^3)</th>
<th>Standard error (r)</th>
<th>Significance(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling site</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left ear</td>
<td>Right ear</td>
<td>0.23 (0.20 to 0.26)</td>
<td>0.02</td>
<td>-0.01 (-0.06 to 0.05)</td>
<td>0.02</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>2nd test</td>
<td></td>
<td></td>
<td>0.04 (-0.01 to 0.09)</td>
<td>0.02</td>
<td>0.14</td>
</tr>
<tr>
<td>Right ear</td>
<td>2nd test</td>
<td>0.23 (0.20 to 0.27)</td>
<td>0.02</td>
<td>0.05 (-0.01 to 0.10)</td>
<td>0.02</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>2nd test(^5)</td>
<td>0.19 (0.16 to 0.21)</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squeezing</td>
<td>No Squeezing</td>
<td>0.19 (0.17 to 0.21)</td>
<td>0.01</td>
<td>-0.06 (-0.10 to -0.02)</td>
<td>0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Squeezing</td>
<td>Squeezing</td>
<td>0.24 (0.21 to 0.28)</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Difference of mean in mmol/L BHBA between sampling location and test results determined at the CCPU
\(^2\) Difference of mean in mmol/L BHBA from the parameter (against the reference test) against the difference in mean in mmol/L from the comparison parameter (against the reference test)
\(^3\) CI\(_{95}\): 95% confidence interval
\(^4\) Adjustment for multiple comparisons by Bonferroni
\(^5\) 2nd test: Second puncture at the ear, approximately 1 cm lateral from the first one at the left or right ear
3.4 ROC analyses

Because of the significant differences in BHBA concentrations found in capillary blood compared with the reference, ROC analyses were performed to determine optimized thresholds for detecting SCK using the FSP-Neo (Table 4). A threshold of 1.2 mmol/L BHBA in serum determined at the CCPU was set to distinguish between animals with SCK and healthy animals. The optimized threshold for using capillary blood was 1.3 mmol/L BHBA for the left and right ear and 1.2 mmol/L BHBA for the repeated testing, either on the left or right ear. Sensitivities for all three sampling sites on the ear were 100%. Specificities ranged between 93% (left ear) and 94% (right ear, repeated measurement). For the sampling site at the left ear, the YI for capillary blood was lowest with 93%, on the other two locations the YI was slightly higher. For all three sampling locations for capillary blood (left, right ear and repeated measurement), the AUC-ROC was 99% with a corresponding Se of 99%, Sp of 94% and a YI of 93%. The optimized threshold for the FSP-Neo using coccygeal blood was 1.1 mmol/L BHBA. The AUC-ROC for coccygeal blood was 100%, with Se and Sp of 100% and 95%, respectively, and YI of 95%. Corresponding test results are presented in Table 4.

The pair wise comparison of the AUC-ROC curves (Figure 4) from the three different capillary sampling locations showed no significant differences; hence, all sampling locations at the ears were suitable to identify dairy cows suffering from SCK.
Table 4: Performances and optimized thresholds of the FSP-Neo to detect SCK in capillary and coccygeal blood based on a threshold for BHBA concentration in serum of 1.2 mmol/L

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FSP-Neo&lt;sup&gt;1&lt;/sup&gt;</th>
<th>capillary blood</th>
<th>coccygeal blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>left ear</td>
<td>right ear</td>
<td>2nd test&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>Optimized threshold&lt;sup&gt;2&lt;/sup&gt; (mmol/L)</td>
<td>1.3</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>AUC-ROC&lt;sup&gt;3&lt;/sup&gt; [%&lt;sup&gt;7&lt;/sup&gt;(CI&lt;sub&gt;95&lt;/sub&gt;)]</td>
<td>99 (98-100)</td>
<td>99 (98-100)</td>
<td>99 (98-100)</td>
</tr>
<tr>
<td>Se&lt;sup&gt;4&lt;/sup&gt; [%&lt;sup&gt;7&lt;/sup&gt;(CI&lt;sub&gt;95&lt;/sub&gt;)]</td>
<td>100 (86-100)</td>
<td>100 (86-100)</td>
<td>100 (86-100)</td>
</tr>
<tr>
<td>Sp&lt;sup&gt;5&lt;/sup&gt; [%&lt;sup&gt;7&lt;/sup&gt;(CI&lt;sub&gt;95&lt;/sub&gt;)]</td>
<td>93 (89-96)</td>
<td>94 (90-97)</td>
<td>94 (89-96)</td>
</tr>
<tr>
<td>YI&lt;sup&gt;6&lt;/sup&gt;</td>
<td>93%</td>
<td>94%</td>
<td>94%</td>
</tr>
</tbody>
</table>

<sup>1</sup> FSP-Neo: FreeStyle Precision Neo (Abbott GmbH & Co. KG, Wiesbaden, Germany)
<sup>2</sup> Based on Receiver Operating Characteristics (ROC) analyses
<sup>3</sup> AUC-ROC: area under the receiver operating characteristics curve
<sup>4</sup> Se: sensitivity
<sup>5</sup> Sp: specificity
<sup>6</sup> YI: Youden Index
<sup>7</sup> CI<sub>95</sub>: 95% confidence interval
<sup>8</sup> 2nd test: Second puncture at the ear, approximately 1 cm lateral from the first one at the left or right ear
<sup>9</sup> All three minimally invasive sampling locations at the ears
Figure 4. ROC curves for four different sampling locations for diagnosis of ketoses either in coccygeal or capillary blood using a serum BHBA concentration of 1.2 mmol/L as threshold for SCK.
3.5 Differences from the reference test

The majority of the 720 capillary blood samples showed a variance of >20% (421 samples, 58%) compared with the reference test. From the 240 coccygeal blood samples showed 208 samples (87%) a variance of ≤20% compared with laboratory results (Table 5).

Table 5: Proportion of samples of the different sampling locations deviating from the results of the reference test considering four different pairs with variance

<table>
<thead>
<tr>
<th>Pairs with variance</th>
<th>Sampling location</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left ear</td>
</tr>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>&lt;5%</td>
<td>24</td>
</tr>
<tr>
<td>5–10%</td>
<td>24</td>
</tr>
<tr>
<td>10–20%</td>
<td>38</td>
</tr>
<tr>
<td>&gt;20%</td>
<td>154</td>
</tr>
<tr>
<td>Total</td>
<td>240</td>
</tr>
</tbody>
</table>

¹ Based on mean of serum BHBA from the CCPU and FSP-Neo test (Abbott GmbH and Co. KG, Wiesbaden, Germany)
² 2nd test: Second puncture at the ear, approximately 1 cm lateral from the first one at the left or right ear
³ All three minimally invasive sampling locations at the ears
3.6 Coefficients of variations

Test results for the repeated measurements are presented in Table 6. The average CV’s for analyzing BHBA concentrations with the FSP-Neo were 2.6% (inter-assay) and 11.7% (intra-assay), respectively. The CV for the reference test used at the CCPU was 1.32% for analysing the BHBA concentrations.

Table 6: Intra-assay and inter-assay coefficients of variation for the FSP-Neo device with low, medium and high BHBA concentrations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.24</td>
<td>1.46</td>
<td>2.23</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>0.05</td>
<td>0.15</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>CV²</td>
<td>22.82</td>
<td>10.39</td>
<td>1.93</td>
<td>11.71</td>
</tr>
<tr>
<td>Inter-assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.20</td>
<td>1.34</td>
<td>2.32</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>0.00</td>
<td>0.05</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>CV²</td>
<td>0.00</td>
<td>4.09</td>
<td>3.61</td>
<td>2.57</td>
</tr>
</tbody>
</table>

1 FSP-Neo: FreeStyle Precision Neo (Abbott GmbH & Co. KG, Wiesbaden, Germany)
2 Coefficient of variation = SD×100/mean
3 Based on reference test values (low = 0.36 mmol/L; medium = 1.30 mmol/L; high = 1.86 mmol/L)
4 Calculated as CV (low + medium + high)/3
4. Discussion

McArt et al. (2015) estimated the cost per case of HYK as approximately $289, hence a rapid and reliable detection of dairy cows that have an elevated BHBA-level is very important from an animal welfare as well as from an economic perspective. Several hand-held meters and other tests have been evaluated in the recent past, using whole blood (Iwersen et al. 2009, Voyvoda and Erdogan 2010, Iwersen et al. 2013, Mahrt et al. 2014b), serum (Iwersen et al. 2013, Pineda and Cardoso 2015), milk (Geishauser et al. 1998, Geishauser et al. 2000, Carrier et al. 2004, Krogh et al. 2011) or urine (Carrier et al. 2004, Krogh et al. 2011) as substrate.

This study provides a systematic evaluation of the use of capillary blood obtained by a minimal invasive lancet technique from an ear of dairy cows to detect HYK. To evaluate the eligibility of this technique, the BHBA concentration in capillary blood was determined at three different sampling locations at the ears by use of an electronic hand-held meter (FSP-Neo). Additionally, test characteristics of the hand-held device for determination of BHBA concentrations in bovine blood were evaluated. For this, the results obtained with the FSP-Neo device at the three different capillary sampling sites and in coccygeal blood samples were compared with the results from coccygeal blood analyzed in the laboratory.

Animals were tested within the first 21 dpp, because the highest incidence for SCK is expected to occur within the first three weeks post partum (Oetzel 2004). The prevalence of SCK in this study (10.4%) was at the lower range compared with recent studies from Europe, that found a prevalence ranging between 11.8% and 39% (Suthar et al. 2013, Berge and Vertenten 2014, Mahrt et al. 2015). Berge and Vertenten (2014) reported that a smaller herd size and feeding a partially instead of a total mixed ration were associated with an increased risk for ketosis. Therefore, the larger herd size and the total mixed ration on this particular farm as well as the farmer’s awareness for HYK (resulting in a continuous progress and implementation of relevant herd health management actions) might be two reasons for this low prevalence.
For the interpretation of results from capillary blood obtained by the FSP-Neo, it is important to evaluate this device by comparing it with a reference standard. For the FSP-Neo device, the Spearman correlation coefficient for coccygeal blood and the laboratory results was $\rho_s = 0.92$. Furthermore, a mean ± SD difference from the reference test of $0.02 \pm 0.21$ mmol/L was found. For an optimal detection of SCK in coccygeal blood with the FSP-Neo device an adjustment of the threshold was made. With an optimized threshold of 1.1 mmol/L, the corresponding Se and Sp were ‘excellent’ with 100% and 95%. Comparing these results with other evaluated hand-held meters, the results were comparable with most devices: Mahrt et al. (2014b) reported a Spearman correlation coefficient for the NovaVet device of $\rho_s = 0.87$ with a mean ± SD BHBA difference of $-0.07 \pm 0.42$ mmol/L compared with the laboratory results. The optimized threshold for the NovaVet device was 1.2 mmol/L with Se and Sp of 97% and 82%. Iwersen et al. (2013) evaluated two different hand-held devices and reported a Spearman correlation coefficient of $\rho_s = 0.94$ with a mean ± SD BHBA difference of $0.04 \pm 0.15$ mmol/L for the FreeStyle Precision device and $\rho_s = 0.80$ with a mean ± SD BHBA difference of $-0.12 \pm 0.22$ mmol/L for the GlucoMen LX Plus hand-held meter. For the FreeStyle Precision at an optimized threshold of 1.2 mmol/L, a similar Se and Sp of 98% and 100% as found in the present study for the FSP-Neo device was reported. For the GlucoMen LX Plus an equal optimized threshold as with the FSP-Neo was reported but a lower Se and Sp with 80% and 86% were determined. For the Optium Xceed (Voyvoda and Erdogan 2010), the Pearson correlation coefficient was $r = 0.97$ with a mean ± SD BHBA difference of $0.04 \pm 0.031$. Voyvoda and Erdogan (2010) performed no adjustment on the threshold and reported a lower Se of 85% and a similar Sp of 94% using a threshold of 1.2 mmol/L for identifying SCK with the Optium Xceed. Considering the results of these and our studies (coefficient of correlation, Bland and Altman plots of differences and ROC-analyses), consistency for BHBA determination with different electronic hand-held meters in different farms with varying environmental conditions can be assumed.

A positive correlation of $\rho_s = 0.76$ to 0.81 was found between the three capillary sampling locations at the ears and the reference test. The mean ± SD BHBA deviation compared with the reference test was $0.20 \pm 0.47$ mmol/L for all three capillary sampling locations. The BHBA concentration in capillary blood analyzed with the FSP-Neo showed a significant
difference compared with the reference test. For practical use, an optimal classification into groups of SCK or healthy cows is more important, therefore adjustments for the threshold were made. To identify SCK in capillary blood obtained from an ear with the FSP-Neo device, an optimized threshold of 1.3 mmol/L should be used. With this threshold, the results from ROC analysis for capillary blood were excellent; overall accuracies were AUC-ROC ≥ 90% with a sensitivity of 100% for all three sampling locations and a specificity of at least 93%. Furthermore, as the pair wise comparison of the AUC-ROC curves from the three capillary sampling locations showed no significant differences, we conclude that the results from all sampling locations at the ears were comparable for the detection of SCK. Kanz et al. (2015) evaluated the use of different hand-held meters for BHBA testing in capillary blood obtained from the skin of the exterior vulva. For the FreeStyle Precision and the NovaVet device, similar Spearman correlation coefficients of $\rho_s = 83\%$ and $\rho_s = 73\%$ with lower mean ± SD BHBA differences of $0.08 ± 0.19$ mmol/L and $-0.01 ± 0.43$ mmol/L, respectively, were described. In that study, an optimized threshold for the FreeStyle Precision of 1.0 mmol/L for detecting SCK, with corresponding Se of 100% and Sp of 76%, was reported. For the NovaVet device the optimized threshold was 1.1 mmol/L with corresponding Se and Sp of 89% and 84%.

The reason for the greater variation for BHBA concentrations measured with the FSP-Neo device in capillary blood than in coccygeal blood compared with the reference test remains speculative. Compared with the reference test, the Bland and Altman plots showed that the measured BHBA concentrations were systematically higher in capillary blood. Therefore, optimized thresholds for identifying SCK in capillary blood were determined. The ROC analyses showed, when these optimized thresholds were applied, that the FSP-Neo device is suitable to detect SCK in capillary blood of dairy cows.

As the Bland and Altman plots showed only slight differences between the three capillary sampling locations at the ears, the results of the mixed model analyses confirmed that the sampling site had no influence on the measured BHBA concentration. Hence, we conclude that all three sampling locations at the ear are suitable for ketone testing.
Another objective of this study was to evaluate the influence of squeezing the skin, in order to get an adequate amount of capillary blood for the measurement, on the determined BHBA concentration. The mean BHBA concentration from the two groups (no squeezing/squeezing) showed a statistical significant difference of 0.06 mmol/L compared with the reference. From a clinical perspective, however, this impact of squeezing is negligible for BHBA measurements using the hand-held device.

In the mixed model analyses of this study, dairy cows in the third or higher lactation showed significantly greater BHBA concentrations compared with cows in the second lactation (no first lactating cows were tested in this study). This finding is in line with previous studies indicating that cows in the first and second lactation had lower prevalence of SCK compared with older cows (Duffield et al. 1998, Mahrt et al. 2015). Furthermore, cows in our study showed greater BHBA concentrations in the first week of lactation compared with cows in the third week of lactation. This finding is line with a study from Duffield et al. (1998), in which the peak risk for SCK was reported during the first two weeks after calving. Mahrt et al. (2015) reported that the risk period for HYK for dairy cows (independent from the lactation number) lasts at least for the first 42 DIM with the highest mean prevalence of 14.6% in lactation week 5.5 and the lowest mean prevalence with 9.6% in lactation week 0.5 and 2.0. These findings were different to the study of McArt et al. (2012), reporting a peak prevalence for SCK at 5 DIM.

Deviations occurred when comparing the differences in the BHBA concentrations between the sampling sites at the ears with the reference test. More than a half of all 720 measurements for capillary blood showed a deviation in the BHBA concentration of more than 20% in comparison with the reference. The measurements in the coccygeal blood using the hand-held device seem to be more accurate, because only 13% showed a deviation in the BHBA concentration of more than 20% compared with the reference test. However, is should be taken into account that the results for capillary blood from the ear were systematically higher than BHBA concentrations from the reference test.
Inter- and intra-assay coefficients of variations were calculated to evaluate the reproducibility and repeatability of the test results. The previously reported average inter- and intra-assay coefficients of variations for another hand-held meter, the FreeStyle Precision device, were 7.2% and 7.9% for capillary blood from the skin of the exterior vulva (Kanz et al. 2015), and 5.8% and 5.7% for whole blood from a tail vessel (Iwersen et al. 2013). The measurements from capillary blood at the ear yielded in similar average inter- and intra-assay coefficient of variation of 2.6% and 11.7%, respectively. Because the CV’s were below 15% as requested from the European Medicines Agency (EMEA, 2011), the results can be considered as good.

Despite the limitations of the study, for instance the low prevalence of SCK on the farm, the detection of HYK in capillary blood from an ear with the FSP-Neo is an option for monitoring SCK under field conditions. Particularly, the excellent test results of the ROC analyses after the adjustments of the thresholds allow discrimination between healthy animals and cows with HYK. Based on the results of our study, additional research including farms with a greater prevalence of SCK, farms without preventing strategies for HYK, and without constant monitoring for HYK is needed to test the external validity of the test results. Because capillary blood is easily achievable from the ear, particularly if animals are fixed in headlocks for routine checkups, this technique is a promising tool for the identification of dairy cows suffering from HYK.
5. Summary

The primary objective of the present study was to test whether capillary blood obtained by puncturing the skin of an ear with a minimal invasive lancet is eligible to detect HYK in dairy cows. To evaluate the eligibility of this technique, the BHBA level of all sampled cows was determined at three different capillary blood sampling locations at the ears. Additionally, test characteristics of a new available hand-held device [FreeStyle Precision Neo (FSP-Neo, Abbott GmbH & Co. KG, Wiesbaden, Germany)] for determination of BHBA concentrations in bovine blood were evaluated by comparing the results with a laboratory reference.

In total, 240 animals within the first 21 dpp were enrolled in the study. The prevalence of SCK was 10.4%. The BHBA concentration was determined with the FSP-Neo device in 720 capillary blood samples from three different sampling sites (left, right ear and repeated measurement either at the left or right ear) and in 240 samples from the coccygeal vessels. The concentration of BHBA in serum harvested from the coccygeal blood samples were analyzed at the Central Clinical Pathology Unit (CCPU), University of Veterinary Medicine, Vienna and were used as the reference test.

The Spearman correlation coefficient ρs between the BHBA concentrations in capillary blood measured with the hand-held device and the reference test was between 0.76 and 0.81. Using capillary blood, the mean ± SD BHBA difference compared with the reference test was 0.20 ± 0.47 mmol/L for all three sampling locations at the ears. The ROC analyses for the FSP-Neo device resulted in an optimized threshold for the detection of SCK in capillary blood of 1.3 mmol/L (left and right ear) and 1.2 mmol/L (second test). Applying these adjusted thresholds, overall accuracies of the results obtained from the ear were ‘excellent’ (AUC-ROC ≥ 90%) compared with the reference test. Sensitivities (Se) for all three capillary sampling sites at the ear were 100%. Specificities (Sp) ranged between 93% (left ear) and 94% (right ear, repeated measurement). The pair wise comparison of the AUC-ROC curves from the three different sampling locations at the ear showed no significant differences, hence, we conclude that all sampling locations were suitable to identify cows suffering from SCK.
The reference test compared with coccygeal blood resulted in $p$-value = 0.92. Using coccygeal blood, the mean $\pm$ SD BHBA deviation compared to the reference test was $0.02 \pm 0.21$ mmol/L. The ROC analyses for the FSP-Neo device resulted in an optimized threshold for the detection of SCK in coccygeal blood of 1.1 mmol/L. The AUC-ROC was 100% for coccygeal blood and the corresponding Se and Sp were 100% and 95%.

Because capillary blood is easily achievable from the ear, particularly if animals are fixed in headlocks for routine checkups, this technique is a promising tool for the identification of dairy cows suffering from HYK. Further confirmation with more results from different farms is required.
6. Zusammenfassung

Das primäre Ziel der Studie war es, die Eignung von am Ohr gewonnen Kapillarblut zur Bestimmung einer Hyperketonämie bei Milchkühen zu testen. Um die Tauglichkeit dieser Technik zu evaluieren, wurde bei allen getesteten Milchkühen die BHBA Konzentration im Kapillarblut an drei verschiedenen Stellen am Ohr bestimmt. Auch die Eignung des neuen elektronischen Schnelltestgerätes [FreeStyle Precision Neo (FSP-Neo, Abbott GmbH & Co. KG, Wiesbaden, Deutschland)] zur Bestimmung der BHBA Konzentration wurde überprüft.


Die Spearman Korrelationskoeffizienten ρs zwischen den BHBA Konzentrationen der Kapillarblutproben, die mit dem FSP-Neo gemessen wurden, und dem Referenzwert betrugen zwischen 0,76 und 0,81. Für alle drei Kapillarblutentnahmestellen am Ohr betrug die durchschnittliche Abweichung 0,20 ± 0,47 mmol/L im Vergleich zum Laborergebnis. Die ROC-Analysen für das FSP-Neo Schnelltestgerät ergaben optimierte Schwellenwerte für das Erkennen einer subklinischen Ketose von 1,3 mmol/L (linkes und rechtes Ohr) und 1,2 mmol/L (Wiederholungstest) für das Kapillarblut. Die Genauigkeit der Testergebnisse der vom Ohr gewonnen Kapillarblutproben waren für die optimierten Schwellenwerte ‘exzellent’ (AUC-ROC ≥ 90%). Die Sensitivitäten (Se) für alle drei Probenentnahmestellen am Ohr betrugen demnach 100%. Die Spezifitäten (Sp) lagen zwischen 93% (linkes Ohr) und 94% (rechtes Ohr, Wiederholungsmessung). Der paarweise Vergleich der AUC-ROC Kurven der drei Probenentnahmestellen am Ohr zeigte keine signifikanten Unterschiede in der Erkennung einer subklinischen Ketose. Die in der Studie verwendeten Punktionsstellen zu Gewinnung von Kapillarblut sind alle geeignet, um eine subklinische Ketose bei Milchkühen zu erkennen.
Der ρr-Wert zwischen dem Referenztest und den mittels FSP-Neo ermittelten BHBA Konzentrationen der Coccygealblutproben war 0,92. Die durchschnittliche Differenz zwischen Coccygealblut und dem Referenztest betrug 0,02 ± 0,21 mmol/L. Die ROC-Analyse für das FSP-Neo Schnelltestgerät ergab einen optimierten Schwellenwerte für das Erkennen einer subklinischen Ketose von 1,1 mmol/L für das Coccygealblut. Die Fläche unter der ROC-Kurve (AUC-ROC) betrug 100% mit einer Se von 100% und einer Sp von 95%.

Da Kapillarblut am Ohr leicht gewonnen werden kann, vor allem wenn Kühe für Routinediagnostik im Fressgitter fixiert sind, ist diese Technik eine vielversprechende Methode zur Identifizierung von Milchkühen die an einer Hyperketonämie leiden. Eine Überprüfung der Studienergebnisse in weiteren Betrieben sollte jedoch erfolgen.
List of abbreviations

SCK  subclinical ketosis
BHBA  β-hydroxybutyrate
dpp  days post partum
DIM  days in milk
HYK  hyperketonemia
FSP-Neo  FreeStyle Precision Neo
CV  coefficients of variations
ρₖ  Spearman correlation coefficient
Se  sensitivity
Sp  specificity
YI  Youden Index
SD  standard deviation
ROC  receiver operating characteristics
AUC-ROC  area under the ROC curve
CCPU  Central Clinical Pathology Unit
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Table 4. Performances and optimized thresholds of the FSP-Neo to detect SCK in capillary and coccygeal blood based on a threshold for BHBA concentration in serum of 1.2 mmol/L

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Figure 3a. Bland-Altmann Plot of differences between BHBA concentrations obtained at the laboratory in serum and measured in capillary blood at three different capillary sampling locations with the FSP-Neo. The upper and lower solid lines illustrate the mean ± 2 SD, the solid line in the middle shows the mean

Figure 3b. Bland-Altmann Plot of differences between BHBA concentrations obtained at the laboratory in serum and measured in coccygeal blood with the FSP-Neo. The upper and lower solid lines illustrate the mean ± 2 SD, the solid line in the middle shows the mean

Figure 4. ROC curves for four different sampling locations for diagnosis of ketoses either in coccygeal or capillary blood using a serum BHBA concentration of 1.2 mmol/L as threshold for SCK