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Evaluation of factors potentially influencing hyperketonemia testing in dairy cows

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Meiner Familie

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1 INTRODUCTION

Negative energy balance (**NEB**) is a common condition in early lactating dairy cows (Bauman and Currie, 1980; Goff and Horst, 1997). Energy demands increase due to the onset of lactation and often cannot be met by dry matter intake (Goff and Horst, 1997). Subsequently, the rate of lipolysis exceeds the rate of lipogenesis in body fat reserves and non-esterified fatty acids (**NEFAs**) are released into the blood (McNamara, 1991). After being taken up by the liver, NEFAs in varying proportions either are oxidized completely, reesterified and stored as triglycerides, removed from the liver in very low-density lipoproteins or metabolized to ketone bodies (Krebs, 1966; Herdt, 2000). In cases of metabolic maladaptation to the NEB, increased circulating concentrations of NEFAs and ketone bodies, i.e. acetone, acetoacetate and ß-hydroxybutyric acid (**BHBA**), can be found in blood, urine and milk (Andersson, 1988; Herdt, 2000).

Hyperketonemia is a common condition in early lactating dairy cows with a prevalence of 20% in cows from 2 to 15 days in milk (**DIM**) in Germany (Suthar et al., 2013). Cows affected by hyperketonemia have increased risks to develop further diseases. Impacts were found on the occurrence of displaced abomasum (**DA**), metritis, clinical ketosis and lameness (Duffield et al., 2009; Suthar et al., 2013). Furthermore, hyperketonemia negatively impacts reproduction (Walsh et al., 2007a, 2007b), increases the risk of culling within the first 60 DIM (Roberts et al., 2012) and, in summary, causes economic losses (Duffield, 2000). Effects of hyperketonemia on milk yield have been described contradictory (Duffield et al., 2009; Chapinal et al., 2012a).

Depending on the presence of clinical signs of ketosis, e.g. loss of appetite, hard dry feces, rapid milk loss or nervous signs (Duffield, 2000), hyperketonemia can be subdivided into clinical ketosis (**CK**) and subclinical ketosis (**SCK**). However, as stated by Gordon et al. (2013), detection of cows showing single signs of clinical ketosis has become difficult in modern housing systems with large cow groups in freestall barns.

The gold standard to diagnose hyperketonemia is measurement of blood or serum BHBA concentrations in laboratories (Oetzel, 2004; McArt et al., 2013). Frequently used thresholds are BHBA concentrations of 1.2 mmol/L to 1.4 mmol/L (Oetzel, 2004; LeBlanc et al., 2005; Duffield et al., 2009). As measurement of BHBA in laboratories is associated with a delay in diagnosis and treatment of hyperketonemia, cow side ketone body tests can provide practical benefits (McArt et al., 2013). A range of semiquantitative milk or urine tests is

available, measuring acetoacetate or BHBA in milk or acetoacetate in urine (Geishauser et al., 1998; Geishauser et al., 2000; Carrier et al., 2004). Presence of ketone bodies results in a chemical reaction leading to a color change of the test, semiquantitatively detectable by the user (Geishauser et al., 1998; Carrier et al., 2004). However, inaccurate results are possible due to subjective interpretation of this color change (Carrier et al., 2004). In the last years, electronic handheld meters, previously used in human medicine, have been evaluated for the use in dairy cows, measuring BHBA in whole blood (Iwersen et al., 2009; Iwersen et al., 2013). Providing quantitative values instantly and without efforts in centrifugation, cooling and transport is a major advance for practical use. Recently, an electronic handheld meter, developed for the use in dairy cows, has become commercially available. However, this handheld meter has not been scientifically evaluated yet.

Several studies found diurnal patterns or effects of sampling time relative to feeding on concentrations of blood BHBA (Plaizier et al., 2005; Nikkhah et al., 2008; Quiroz-Rocha et al., 2010). However, during these studies, cows were fed only once or twice daily. There is a lack of information on diurnal patterns of BHBA concentration under conditions of a continuous total mixed ration (**TMR**) feeding regimen and how sampling time and frequency of testing affect test characteristics of BHBA measurements.

Another determinant potentially influencing BHBA concentrations is the blood vessel chosen for sampling (Oetzel, 2004). As there is a mammary uptake of BHBA from the blood (Kronfeld et al., 1968), blood samples for BHBA measurement should not be collected from the mammary vein (V. epigastrica cranialis superficialis) (Oetzel, 2004). Redetzky et al. (2003) compared BHBA concentrations measured in blood obtained from the mammary vein and the jugular vein (V. jugularis). Wilhelm et al. (2013) additionally examined the saphenous veins as a blood sampling location for BHBA measurement. Both studies found increased blood BHBA concentrations in samples drawn from the jugular vein compared to the mammary vein in lactating dairy cows, although only Wilhelm et al. (2013) demonstrated a statistical significance. However, the tail vessels (A./V. coccygea), an in scientific research and daily practice frequently used blood sampling location (Iwersen, 2009, 2013; McArt, 2012a), have not been considered in both of these trials.

To evaluate the metabolic situation of dairy herds and to detect affected cows, routine monitoring programs are necessary (Ospina et al., 2013). Treatment of individual cows detected as hyperketonemic is beneficial (McArt et al., 2014). Early lactating, hyperketonemic cows treated with 300mL propylene glycol as a drench once daily until BHBA concentration declined under 1.2 mmol/L were less likely to develop a DA, to be sold or to die than

untreated control cows (McArt et al., 2012b) and were more likely to resolve their hyperketonemia (McArt et al., 2011).

Beside the identification and treatment of individual cows affected, the second objective of hyperketonemia screening programs is to evaluate and monitor the success of current management and feeding protocols and to notice deviations (LeBlanc, 2010). Results of repeated screenings can provide 2 main characteristics: incidence and prevalence of hyperketonemia (McArt et al., 2013). Prevalence can be determined by conducting a single cross-sectional sampling and subsequent dividing the number of cows affected by hyperketonemia by the total number of cows sampled (Ospina et al., 2013). Proportions of 10% (Oetzel, 2004) to 25% (Chapinal et al., 2012b) of cows with elevated BHBA concentrations (≥ 1.4 mmol/L) have been described to be appropriate herd alarm levels. However, describing the prevalence of hyperketonemia, these thresholds only represent a snap shot of the situation (McArt et al., 2013).

In contrast, determination of incidence requires frequent measurements during the entire risk period (McArt et al., 2013). Incidence is calculated by dividing the number of cows diagnosed with hyperketonemia for the first time during the observation period by all cows at risk. Cows are no longer at risk after once been diagnosed as hyperketonemic (McArt et al., 2013). Sampling frequency should depend on the median duration of the disease observed (McArt et al, 2013). The latter authors (McArt et al., 2012a) found a median duration of SCK of 5 days, therefore a biweekly sampling scheme is needed to estimate incidence (Ospina et al., 2013).

Although the prevalence is easier to determine, knowing a herd´s incidence has benefits in optimizing management, testing and treatment protocols and enables the accurate identification of cows suffering from hyperketonemia (McArt et al., 2013). On the other hand, a hyperketonemia screening protocol should not exceed a manageable effort, should be cost-effective and implementable in daily routine practice. Therefore, for planning an effective hyperketonemia screening protocol, precise information on factors potentially influencing measurement of BHBA is necessary. Potential sources of error in herd testing are related to the selection of animals and sampling time frame during early lactation. Sampled animals should be at risk, but subjectively healthy to obtain information on incidence and prevalence of hyperketonemia (Ospina et al., 2013). A frequently used sampling time frame for hyperketonemia testing is approximately the first 2 weeks postpartum (Duffield et al., 2009; McArt et al., 2012a; Ospina et al., 2013). Longer risk periods have been mentioned by Andersson (1988), Dohoo and Martin (1984) and Holtenius and Holtenius (1996). Dairy

husbandry and feeding approaches, however, have changed during the last decades and there is a lack of systematic research on the time of onset and duration of hyperketonemia in early lactation dairy cows under modern husbandry conditions.

The overall objective of this thesis, including 4 corresponding studies, therefore was to evaluate factors potentially influencing hyperketonemia testing in early lactation dairy cows.

In particular, 1) the effect of sampling time on BHBA concentrations in continuously fed dairy cows was studied, test characteristics for a single BHBA measurement at a random time of the day to diagnose hyperketonemia were calculated and evaluated if these can be enhanced by repeating measurement after several time intervals.

Furthermore, 2) the effects of different blood sampling locations, including the tail vessels, on concentrations of BHBA were tested.

In a third approach, 3) time of onset and duration of hyperketonemia in frequently tested dairy cows was observed and test characteristics of different hyperketonemia testing protocols during the first 42 DIM determined.

In a fourth approach, 4) an electronic handheld meter, measuring blood concentrations of BHBA, was evaluated for the use in dairy cows.

2 RESEARCH PAPERS

2.1 Effects of time and sampling location on concentrations of ß-hydroxybutyric acid in dairy cows

A. Mahrt, O. Burfeind, and W. Heuwieser. 2014. J. Dairy Sci. 97:291-298

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2.2 Evaluation of hyperketonemia risk period and screening protocols for early-lactation dairy cows

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2.3 Evaluation eines neuen elektronischen Handmessgeräts zur Messung von ß-Hydroxybutyrat bei Milchkühen

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The manuscripts of the papers are formatted according to the guide for authors of the respective journal.

TIMING AND SAMPLING LOCATION FOR BHBA MEASUREMENT

2.1 Effects of time and sampling location on concentrations of ß-hydroxybutyric acid in dairy cows

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SCREENING DAIRY COWS FOR HYPERKETONEMIA

2.2 Evaluation of hyperketonemia risk period and screening protocols for early-lactation dairy cows

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Evaluation of a new electronic handheld meter for measurement of ß-hydroxybutyric acid in dairy cows

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Diesen Teil müssen Sie online erwerben.

3 DISCUSSION

The overall objective of this thesis was to evaluate factors potentially influencing hyperketonemia testing in early lactating dairy cows. The results of the first study indicate that sampling continuously TMR fed dairy cows for measurement of BHBA can be performed at any time of the day to diagnose hyperketonemia. Repeating measurements after several time intervals increases test characteristics only slightly compared to a single measurement at a random time of the day, which shows very good test characteristics. However, it is important to notice that these results only refer to frequently fed cows. They are therefore particularly useful in conveyer belt or automatic feeding system fed herds, where feeding frequencies usually are high (Belle et al., 2012). DeVries et al. (2005) found an increased feed delivery frequency to increase the feeding time as well as the distribution of feeding time over the course of the day. Quiroz-Rocha et al. (2010) observed fluctuating BHBA concentrations in cows fed a TMR, in contrast, only once or twice daily. They found BHBA significantly increasing between 4 and 10 h after the first feeding. This finding might be explained by a postprandial increase in alimentary ketogenesis, i.e. butyrate absorption and subsequent conversion to BHBA by the rumen epithelium (Reynolds, 2002).

The second factor potentially influencing hyperketonemia testing studied in this thesis was the blood vessel used for sampling. Frequently used blood sampling locations in dairy cows are the jugular vein (V. jugularis), the mammary vein (V. epigastrica cranialis superficialis) and the tail vessels (A./V. coccygea) (Windisch et al., 1991; McArt et al., 2012b; Laeger et al., 2013). The jugular vein and mammary vein have been compared considering BHBA concentration (Redetzky et al., 2003; Wilhelm et al., 2013). Blood BHBA concentration from samples drawn from the jugular vein was significantly higher than from samples drawn from the mammary vein in lactating dairy cows (Wilhelm et al., 2013). The authors furthermore included the Vv. saphenae externae in the comparison, where they found higher concentrations of BHBA than in samples drawn from the jugular vein (Wilhelm et al., 2013). They supposed that this difference might be due to a different ketone body metabolism of muscle and brain tissue. However, the A./V. saphena are inconvenient as blood sampling locations in dairy cows in daily practice due to a high demand on restraint (Nagy et al., 2002). In contrast, the tail vessels are frequently used for BHBA measurement in practice and multiple studies (van der Drift et al., 2012; Iwersen et al., 2013; Denis-Robichaud et al.,

2014). Although it is known that the blood sampling location chosen for measurement has an impact on BHBA concentrations, an evaluation of the tail vessels as sampling location for BHBA measurement to our knowledge has not been published yet. Ketone bodies are used as energy source by several tissues (Heitmann et al., 1987) and serve as milk fat precursors in the lactating mammary gland (Delamaire and Guinard-Flament, 2006). The arterial BHBA concentration is the major determinant of BHBA uptake by the mammary gland (Miller et al., 1991) and arteriovenous differences in lactating dairy cows are well documented (Kronfeld et al., 1968; Delamaire and Guinard-Flament, 2006; Guinard-Flament et al., 2011). However, as other tissues like brain and skeletal muscle likewise are able to use BHBA as an energy source (Robinson and Williamson, 1980; Laeger et al., 2010), a potential impact on BHBA concentrations in different blood vessels cannot be ruled out.

The data show that blood BHBA concentrations differ comparing the 3 sampling locations mammary vein, jugular vein and tail vessels. Significant differences were found between the mammary vein and both the jugular vein and the tail vessels. Concentrations of BHBA did not differ significantly between the jugular vein and tail vessels. Consequently, this impact should be considered and blood samples for measurement of BHBA should not be drawn from the mammary vein, but from the jugular vein or tail vessels.

Effective identification of cows suffering from hyperketonemia requires reliable information on occurrence patterns of this disease during early lactation. The aim of the third study therefore was to examine hyperketonemia occurrence patterns in dairy cows during the first 6 weeks of lactation.

As a main result, the study shows that hyperketonemia affects cows during the entire examination period until 42 DIM. Testing cows solely during the first 2 weeks of lactation, as frequently recommended (McArt et al., 2014; Ospina et al., 2013), would have missed 47% of all cows affected during this study. There is evidence for the existence of 2 different main types of hyperketonemia, differing in etiology and time of occurrence (Holtenius and Holtenius, 1996; Herdt, 2000; Ingvartsen, 2006). Type I ketosis occurs 3 to 6 weeks postpartum in high yielding cows, where the demand for glucose exceeds the glucose production of the liver due to a lack of substrate. Type II ketosis occurs earlier in lactation and is accompanied by varying degrees of fatty liver. Cows affected by type II ketosis often suffer from other diseases like mastitis or metritis. A predisposing factor is overfeeding during the dry period (Holtenius and Holtenius, 1996). Cows affected by type II ketosis, however, may stay hyperketonemic until lactation week 3, therefore the etiologic assignment of

hyperketonemia solely depending on the time of occurrence seems to be difficult (Oetzel, 2004).

Effects of hyperketonemia occurring later than 3 weeks postpartum on disease risk as well as corresponding herd alarm levels have not been evaluated thoroughly. Walsh et al. (2007b) found non pregnant cows tending to have increased BHBA concentrations from 3 weeks ante partum until 9 weeks postpartum compared to cows pregnant after first artificial insemination. Hyperketonemia at any time within the first 5 weeks of lactation was associated with a decreased 6-week pregnancy rate in predominantly pasture-grazed dairy cows (Compton et al., 2015). Most other studies investigating the impact of hyperketonemia on disease risk and reproductive performance considered only BHBA concentrations measured during the first 1 to 3 weeks postpartum (LeBlanc et al., 2005; Walsh et al., 2007a; Duffield et al., 2009; Chapinal et al., 2011, 2012a; Roberts et al., 2012; Suthar et al., 2013). Increased BHBA concentrations, however, have been found to affect parameters of the immune system in vivo and in vitro in cows later in lactation, too (Suriyasathaporn et al., 1999; Zarrin et al., 2014). Effects of hyperketonemia occurring later in lactation on disease risk therefore cannot be excluded.

Evaluating the number of positive BHBA test results during the first 42 DIM, the study showed that a large part of all hyperketonemic cows had only 1 positive test during the whole observation period and recovered without any treatment. This is a new finding and raises the question of whether these cows have the same risk for disease and negative effects on reproductive performance as cows suffering from longer lasting or recurring hyperketonemia. Walsh et al. (2007b) found a dose-response relationship between the probability and median time to pregnancy and the duration of hyperketonemia during the first 2 weeks of lactation, but there is a lack of research on effects of the duration of hyperketonemia during the first 42 DIM on disease risk. To consider upcoming research findings concerning this matter, 2 different gold standard definitions for hyperketonemia were used for the subsequent evaluation of different hyperketonemia screening protocols, distinguishing between cows experiencing only a single (G1) or at least 2 positive hyperketonemia tests (G2) during the observation period.

To determine the capability of different hyperketonemia testing protocols, test characteristics (sensitivity, specificity, positive and negative predictive values) of different protocols containing single or repeated BHBA measurements during the first 6 weeks of lactation were calculated. Test characteristics improved with the number of BHBA measurements, which was to be expected. Sampling cows weekly resulted in a sensitivity of

91% and a specificity of 83% considering cows suffering from a distinct, continuing or recurring hyperketonemia, indicated by at least 2 positive BHBA test results during the observation period. Positive and negative predictive values were 68% and 96%, respectively. The poorer results for specificity and positive predictive value of this protocol resulted from cows with only 1 positive BHBA test during the observation period, that are correctly diagnosed as hyperketonemic at the particular test day, but do not achieve the gold standard (at least 2 positive tests). The screening protocol therefore identifies a large majority of cows affected by continuing or recurring hyperketonemia and enables their treatment as well as a close monitoring of feeding and management procedures. On the other hand, it assumes a relatively high level of effort and overestimates the existence of distinct hyperketonemia due to the detection of cows with only 1 positive BHBA test. In contrast, a biweekly screening protocol, likewise considering only cows with continuing or recurring hyperketonemia, resulted in a sensitivity of 69% and a specificity of 91%. Positive and negative predictive values were 75% and 88%, respectively. Using this protocol, less cows are diagnosed false positive, whereas a certain proportion of affected cows will be missed.

McArt et al. (2015) most recently analyzed the costs associated with hyperketonemia. Average costs per case of hyperketonemia were $117, considering costs of diagnostics using a handheld BHBA meter, therapeutics, labor, milk losses, death losses, culling and reproduction losses. Diagnostics, labor and therapeutics accounted for only 6% of these costs. Considering additional costs caused by hyperketonemia-attributed diseases, the average total costs per case of hyperketonemia were $289. Although these values are calculated for North American dairy farm and market conditions, like for example regarding the milk, feed and market cow prices, they illustrate that diagnostics and therapeutics account for only a small part of the costs. In a second publication, McArt et al. (2014) developed economic models to evaluate different hyperketonemia testing and treatment schemes. The models included BHBA testing schemes with varying test intervals between 3 to 16 DIM and a treatment containing daily oral administrations of 300 mL propylene glycol for 5 days. This therapy was administered as blanket treatment or after diagnosis of hyperketonemia. Mean benefits of the different testing and treatment schemes considering a herd with a hyperketonemia incidence of 40% during the first 30 DIM were, depending on the particular scheme, $744 to $1166 per 100 fresh cows. In contrast to these testing schemes, the screening protocols evaluated here include an extended period during early lactation. Negative effects of hyperketonemia during the first 5 weeks of lactation on reproductive performance are proven (Compton et al., 2015) and a negative impact on disease risk cannot be excluded. Furthermore, costs for diagnostic and treatment of

hyperketonemia are relatively low, compared to the benefits of a treatment with propylene glycol (McArt et al., 2014; 2015). Hyperketonemia screening protocols therefore should consider the first 6 weeks of lactation and include at least a biweekly testing.

The objective of the fourth study was to evaluate an electronic handheld blood BHBA meter (NovaVet, Nova Biomedical, Waltham, USA) for the use in dairy cows. The diagnosis of hyperketonemia, particularly in cases of subclinical ketosis, solely depends on measurement of ketone body concentrations in blood, milk or urine (Andersson, 1988). Requirements on cow-side hyperketonemia tests therefore include good test characteristics (sensitivity, specificity, positive and negative predictive values) compared to the gold standard as well as a time-, labor- and cost-effective measurement. Different semiquantitative cow-side hyperketonemia test strips, tablets or powders are commercially available and have been evaluated with varying results (Geishauser et al., 1998, 2000; Enjalbert et al., 2001; Carrier et al., 2004; Iwersen et al., 2009). Concentrations of either acetoacetic acid in urine or acetoacetic acid or BHBA in milk are indicated by color change. Subjective assessment of the degree of this color change is a main disadvantage in the use of these tests (Carrier et al., 2004). Urine samples could not be obtained out of every cow during evaluation of these tests (Carrier et al., 2004; Iwersen et al., 2009), which also might represent a problem in daily practice. Milk ketone test results are affected by somatic cell count (Geishauser et al., 2000). Over the last years, different electronic handheld BHBA meters, developed for the use in human medicine, have been evaluated for the use in dairy cows considering BHBA thresholds of 1.2 to 1.4 mmol/L (Iwersen et al., 2009, 2013; Voyvoda and Erdogan, 2010). Sensitivities of these devices varied between 80% to 100%, whereas specificities were 87% to 100%. All of these BHBA handheld meters have been considered to be useful for monitoring hyperketonemia in dairy cows (Iwersen et al., 2009, 2013; Voyvoda and Erdogan, 2010).

The aim of this study was to evaluate test characteristics of a new commercially available BHBA meter (NovaVet), developed for the use in dairy cows. Sensitivity of this device was, depending on the serum BHBA threshold used as gold standard, 91% to 97% and thus higher than or comparable to sensitivities determined for other electronic BHBA handheld meters. Specificity, however, was 82% to 89% and thus lower than calculated for other devices. Negative predictive values of the NovaVet meter were, depending on the particular BHBA threshold, 98% to 99%, which means that the large majority of cows diagnosed as non hyperketonemic truly were not suffering from hyperketonemia. In contrast, the positive predictive values were 52% to 59%, which means that a considerable proportion

of cows were wrongly diagnosed as hyperketonemic during the evaluation, calculated for a hyperketonemia prevalence of 21.3% (BHBA ≥ 1.2 mmol/L). As the predictive values, however, strongly depend on the prevalence of a condition (Altman and Bland, 1994), these characteristics are not consistent and may vary between farms. Furthermore, costs for diagnostics, labor and therapeutics of hyperketonemia represent only a very small part of the total costs of a case of hyperketonemia (McArt et al., 2015). The NovaVet BHBA meter, in summary, can be recommended for the use in dairy cows under consideration of a certain degree of overestimation of the prevalence of hyperketonemia.

Altogether, the results of the studies indicate that a single sampling of continuously TMR fed dairy cows for BHBA measurement at a random time of the day provides very good test characteristics for on farm conditions. Furthermore, the V. jugularis or A./V. coccygealis should be used as blood sampling location for BHBA measurement in lactating cows. Screening programs for hyperketonemia should include at least the first 42 DIM. A biweekly testing scheme achieves a sensitivity of 69% and a specificity of 91% considering cows with a distinct hyperketonemia. The NovaVet blood BHBA meter can be used for the diagnostic of hyperketonemia in dairy cows, however, a certain number of false positive results must be considered.

4 SUMMARY

Annika Mahrt: Evaluation of factors potentially influencing hyperketonemia testing in dairy cows

Hyperketonemia is a common condition in early lactating dairy cows. Usually induced by a maladaptation to the negative energy balance affecting most dairy cows during early lactation, it increases the risk for the development of subsequent diseases. Furthermore, a negative impact on reproduction parameters is proven. As most cows suffering from hyperketonemia do not show clinical signs of ketosis, the diagnosis depends on the measurement of ketone bodies in blood, milk or urine. Laboratory-based measurement of increased blood concentrations of ß-hydroxybutyrate (**BHBA**) is the gold standard for the diagnosis of hyperketonemia, using cut-points of 1.2 mmol/L or 1.4 mmol/L. In addition, a range of semiquantitative or quantitative cow-side hyperketonemia tests is available. Currently, electronic handheld blood BHBA meters are frequently used in the diagnostic of hyperketonemia.

Strategic hyperketonemia screening protocols have been recommended, addressing 2 main objectives: first to detect and subsequently treat affected cows and second to monitor and evaluate the success of early lactation feeding and management procedures. The overall objective of this thesis was to evaluate factors potentially influencing the diagnostic of hyperketonemia in dairy cows.

The aim of the first study was to investigate the effect of sampling time in the course of a day on blood BHBA concentrations in continuously fed dairy cows. Furthermore, test characteristics of single and repeated BHBA measurements during the day to diagnose subclinical ketosis were determined for 2 frequently used BHBA cut-points (1.2 mmol/L and 1.4 mmol/L). A total of 128 cows (8 to 28 days in milk (**DIM**) fed with a total mixed ration (**TMR**) 10 times daily were preselected by blood BHBA concentration, measured with an electronic handheld meter. Cows with BHBA concentrations $\geq$ 0.8 mmol/L at the initial measurement were enrolled in the trial (n = 92). Subsequent measurements were repeated in intervals of 3 h for a total of 8 measurements during 24 h.

The analysis conducted indicated that sampling time did not affect BHBA concentration (P = 0.23). To evaluate test characteristics of single and repeated BHBA

measurements, the average daily BHBA concentration, averaged from the 8 measurements, was used as gold standard. A single BHBA measurement at a random time of the day had a sensitivity of 0.90 and a specificity of 0.88, using a BHBA concentration of 1.2 mmol/L as cut-point. Using 1.4 mmol/L as cut-point, sensitivity and specificity were 0.89 and 0.90, respectively. Repeating measurements after different time intervals in cases of marginal BHBA concentrations (1.0 mmol/L to 1.6 mmol/L) and using the averaged BHBA concentration from both measurements to diagnose hyperketonemia improved test characteristics only slightly. In conclusion, blood samples for measurement of BHBA in continuously TMR fed dairy cows to can be drawn at any time of the day. A single measurement provides very good test characteristics, which can be enhanced slightly by repeating measurements in cows with marginal BHBA concentrations.

The second study was conducted to compare BHBA concentrations in blood samples drawn from 3 in daily practice frequently used sampling locations (A./V. coccygealis, V. jugularis, V. epigastrica cranialis superficialis). Blood samples of 116 early lactating dairy cows were drawn from the 3 sampling locations and tested for BHBA using an electronic handheld meter. Concentrations of BHBA differed between the sampling locations ($P = 0.03$). Mean BHBA concentration in samples drawn from the V. epigastrica cranialis superficialis. was 0.3 mmol/L lower compared with samples drawn from the V. jugularis and 0.4 mmol/L lower compared with samples drawn from the A./V. coccygealis. Mean BHBA concentration did not differ significantly between samples drawn from the V. jugularis and samples drawn from the A./V. coccygealis ($P = 0.82$). In conclusion, blood samples for measurement of BHBA in lactating dairy cows should be drawn from the V. jugularis or A./V. coccygealis, whereas samples drawn from the V. epigastrica cranialis superficialis.contain significantly lower concentrations of BHBA.

The objective of the third study was to describe factors concerning the timing and setting of hyperketonemia screening protocols during early lactation. A total of 252 cows from 3 dairy farms were tested twice weekly during the first 42 DIM using an electronic handheld BHBA meter. The resulting 12 test results per cow therefore corresponded to lactation weeks 0.5 to 6.0. The time of onset of hyperketonemia, the number of positive hyperketonemia test results, and the duration of the longest hyperketonemic period during the first 42 DIM were investigated. Furthermore, test characteristics of single and repeated BHBA measurements during this period to diagnose hyperketonemia were evaluated to simulate

different hyperketonemia screening protocols. In detail, test characteristics of 4 different testing scenarios (testing all cows 1, 2, 3, or 6 times during the first 42 DIM) were calculated for 2 different gold standard definitions (BHBA ≥ 1.2 mmol/L at least once or BHBA ≥ 1.2 mmol/L at least twice during the first 42 DIM).

Mean prevalence of hyperketonemia was 11.8% and ranged between 9.6% in lactation weeks 0.5 and 2.0 and 14.6% in lactation week 5.5. A total of 134 cows (53.2%) had at least 1 positive hyperketonemia test result during the observation period. Of these cows, 47% had the first positive hyperketonemia test later than 14 DIM. The median first positive hyperketonemia test result occurred in lactation week 2.0 (interquartile range (**IQR**) 1.0 to 3.5). Of all cows with hyperketonemia, 46.3% had only 1 positive test result. Median frequency of positive BHBA test results in cows affected by hyperketonemia was 2 (IQR 1 to 3). The longest hyperketonemic period in affected cows lasted in median 1 examination interval (3-4 days; IQR 1-2 examination intervals).

Test characteristics of the 4 testing scenarios varied depending on the test frequency and the gold standard considered. A single BHBA measurement during the whole 42 day period achieved a sensitivity of 21% and a specificity of 100% considering a minimum of 1 positive hyperketonemia test result during the 42 day period as gold standard. A testing scenario including weekly measurements of BHBA had a sensitivity of 72% and a specificity of 100% considering the same gold standard. Increasing the gold standard to a minimum of 2 positive hyperketonemia test results, i.e. in cases of sustained or repeated hyperketonemia, sensitivity and specificity were 33% and 97% for a single BHBA measurement during the first 42 DIM and 91% and 83% for a testing scenario including weekly measurements, respectively.

These data, in conclusion, show that hyperketonemia affects cows at least over the first 42 DIM. Screening protocols therefore should consider a longer period than the frequently mentioned first 2 weeks of lactation. A moderately laborious screening protocol including a biweekly testing scheme during the first 42 DIM achieves a sensitivity of 69% and a specificity of 91% considering cases of sustained or repeated hyperketonemia. Further research is needed to investigate the effects of an isolated positive BHBA tests during the whole observation period on disease risk, milk yield and reproductive performance.

The objective of the fourth study was to evaluate test characteristics of a new commercially available electronic handheld BHBA meter to diagnose hyperketonemia in dairy cows. Blood BHBA in samples from 155 lactating dairy cows from 3 farms was

measured using the handheld device (NovaVet, Nova Biomedical, Waltham, MA, USA). Subsequently, blood was centrifuged and serum analyzed by a commercial laboratory. Concentrations determined by the device and the laboratory were compared. The methods were highly correlated ($r_s = 0.87$; $P < 0.05$). A difference (median 0.0 mmol/L; IQR -0.1 to 0.2 mmol/L; $P < 0.05$) was observed between BHBA concentrations measured by the handheld device (median 1.0 mmol/L; IQR 0.7 to 1.3 mmol/L) and the laboratory (median 0.9 mmol/L; IQR 0.7 to 1.1 mmol/L). Sensitivity and specificity of the handheld device were 97% and 82%, respectively, using a cut-point of 1.2 mmol/L to diagnose cows as hyperketonemic. Using a gold standard cut-point of 1.4 mmol/L, the receiver operating characteristic analysis determined an adjusted cut-point of 1.3 mmol/L for the handheld device. Sensitivity and specificity were 96% and 85%, respectively. In conclusion, the handheld device can be recommended for the diagnostic of hyperketonemia in dairy cows. However, due to the specificities of 82% and 85%, respectively, a certain number of false positive results must be considered.

5 ZUSAMMENFASSUNG

Annika Mahrt: Evaluation von Einflussfaktoren auf die Diagnostik der Hyperketonämie bei Milchkühen

Ein Großteil der Milchkühe weist während der Frühlaktation erhöhte Ketonkörperkonzentrationen im Blut auf. Diese werden in der Regel durch eine Maladaptation des Metabolismus an die in dieser Zeit sehr häufig auftretende negative Energiebilanz verursacht. Gesicherte Folgen der Hyperketonämie sind eine erhöhte Wahrscheinlichkeit der Kühe, an assoziierten Erkrankungen wie Labmagenverlagerung oder Metritis zu erkranken, sowie eine verminderte Reproduktionsleistung. Da die Hyperketonämie häufig ohne klinische Anzeichen verläuft, beschränkt sich die Diagnostik auf die Messung der 3 Ketonkörper Aceton, Acetoacetat und ß-Hydroxybutyrat (**BHB**) in Blut, Milch oder Urin. Die laborgestützte Messung von BHB im Blut oder Serum ist als Goldstandard zur Diagnose der Hyperketonämie etabliert. Häufig verwendete Grenzwerte sind 1,2 mmol/L und 1,4 mmol/L. Zusätzlich ist eine Anzahl semiquantitativer Testverfahren auf dem Markt, bei denen der Farbumschlag eines pulverförmigen, auf Teststreifen aufgebrachten oder in Tablettenform gepressten Reagens das Vorhandensein erhöhter Ketonkörperkonzentrationen in Milch oder Urin anzeigt. Seit einiger Zeit existieren zusätzlich elektronische Handmessgeräte zur Messung von BHB im Vollblut.

Zum Auffinden an Hyperketonämie erkrankter Tiere sowie zur Überwachung und Erfolgskontrolle von Management- und Fütterungsprotokollen wird der Einsatz strategischer Screeningprogramme empfohlen. Ziel dieser Doktorarbeit war die Evaluation möglicher die Diagnostik der Hyperketonämie beeinflussender Faktoren.

Zielsetzung der ersten Studie war die Untersuchung des Einflusses des Probenentnahmezeitpunktes im Tagesverlauf auf BHB-Blutkonzentrationen bei kontinuierlich gefütterten Kühen. Weiterhin wurden Testcharakteristika einzelner sowie im Tagesverlauf wiederholter BHB-Messungen zur Diagnostik der Hyperketonämie für 2 häufig verwendete Grenzwerte (1,2 mmol/L und 1,4 mmol/L) bestimmt. Einer Gesamtanzahl von 128 frühlaktierenden Kühen (Laktationstag 8 bis 28), denen mittels Hochband zehnmal täglich eine Totale Mischration (**TMR**) vorgelegt wurde, wurden Blutproben entnommen und mittels eines elektronischen Handmessgerätes auf die Höhe der BHB-Konzentration untersucht. Kühe

mit BHB-Konzentrationen ≥ 0,8 mmol/L wurden in die Studie aufgenommen (n = 92). Im weiteren Verlauf erfolgten wiederholte Blutentnahmen in Intervallen von 3 Stunden über eine Gesamtdauer von 24 Stunden.

Die nachfolgende Auswertung ergab, dass der Zeitpunkt der Probenentnahme im Tagesverlauf bei kontinuierlich gefütterten Kühen keinen Effekt auf die Höhe der gemessenen BHB-Konzentration hat (P = 0,23). Zur Evaluation der Testcharakteristika einer einzelnen sowie wiederholter BHB-Messungen im Tagesverlauf wurde für jede Kuh das arithmetische Mittel, gebildet aus den 8 Messwerten, als Goldstandard zur Diagnose einer Hyperketonämie verwendet. Eine einzelne BHB-Messung zu einem zufälligen Tageszeitpunkt wies bei einem Grenzwert von 1,2 mmol/L eine Sensitivität von 0,90 und eine Spezifität von 0,88 auf. Eine Erhöhung des Grenzwertes auf 1,4 mmol/L ergab eine Sensitivität von 0,89 und eine Spezifität von 0,90. Die Wiederholung der Messung nach verschiedenen Zeitspannen und nachfolgende Verwendung des Durchschnittswertes beider Messungen in Fällen, bei denen eine grenzwertnahe BHB-Konzentration (1,0 mmol/L bis 1,6 mmol/L) gemessen wurde, verbesserte die Testcharakteristika nur geringfügig. Daher ist die Entnahme von Blutproben zur BHB-Messung bei kontinuierlich gefütterten Kühen zu jeder Tageszeit möglich. Eine einmalige Messung zur Diagnostik der Hyperketonämie weist sehr gute Testcharakteristika auf, die durch eine zeitlich verzögerte Wiederholung der Probenentnahme und Messung bei Kühen mit grenzwertnahen BHB-Konzentrationen nur geringfügig verbessert werden kann.

Ziel der zweiten Studie war der Vergleich dreier in der Praxis häufig verwendeter Blutentnahmeorte (A./V. coccygealis, V. jugularis und V. epigastrica cranialis superficialis) zur BHB-Messung. Hierzu wurden 116 laktierenden Kühen Blutproben an den 3 Entnahmeorten entnommen und mittels eines elektronischen Handmessgerätes auf die enthaltenen BHB-Konzentrationen hin untersucht. Die gemessenen BHB-Konzentrationen schwankten zwischen den aus den unterschiedlichen Blutgefäßen entnommenen Proben (P = 0,03). Die aus der V. epigastrica cranialis superficialis entnommenen Proben enthielten um 0,3 mmol/L niedrigere mittlere BHB-Konzentrationen als die aus der V. jugularis entnommenen Proben sowie um 0,4 mmol/L niedrigere mittlere BHB-Konzentrationen als die aus der A./V. coccygealis entnommenen Proben. Zwischen den aus der V. jugularis und der A./V. coccygealis entnommenen Proben bestanden keine signifikanten Konzentrationsunterschiede (P = 0,82). Aus diesen Ergebnissen schlussfolgernd sollten Blutproben zur BHB-Messung bei laktierenden Milchkühen aus der V. jugularis oder der

A./V. coccygealis entnommen werden. Aus der V. epigastrica cranialis superficialis entnommene Proben weisen signifikant niedrigere BHB-Konzentrationen auf.

Das Ziel der dritten Studie war die Beschreibung epidemiologischer Eckdaten der Hyperketonämie während der Frühlaktation sowie die Evaluation verschiedener Protokolle zur Erkennung erkrankter Tiere. Im Einzelnen wurde der Zeitpunkt des Beginns der Hyperketonämie, die Anzahl positiver Testergebnisse sowie die Dauer der längsten hyperketonämischen Phase während der ersten 42 Laktationstage untersucht. Weiterhin wurden Testcharakteristika einzelner und wiederholter BHB-Messungen während dieses Zeitraumes zur Diagnose einer Hyperketonämie evaluiert. Hierzu wurden 4 verschiedene Testscenarien (1, 2, 3 oder 6 BHB-Messungen während der ersten 42 Laktationstage) unter Verwendung zweier verschiedener Goldstandard-Definitionen (G1: mindestens ein BHB-Wert ≥ 1,2 mmol/L oder G2: mindestens 2 BHB-Werte ≥ 1,2 mmol/L während der ersten 42 Laktationstage) entwickelt. Insgesamt 252 Milchkühe von 3 Betrieben wurden während der ersten 42 Laktationstage zweimal wöchentlich mit Hilfe eines elektronischen BHB-Messgerätes untersucht. Die resultierenden 12 Testergebnisse pro Kuh entsprachen somit den Laktationswochen 0,5 bis 6,0.

Die Auswertung der Daten ergab eine mittlere Hyperketonämie-Prävalenz von 11,8% mit Werten zwischen 9,6% in den Laktationswochen 0,5 und 2,0 und 14,6% in Laktationswoche 5,5. Insgesamt wiesen 134 Kühe (53,2%) zu mindestens einem Untersuchungszeitpunkt eine Hyperketonämie auf. Bei 47% dieser Kühe trat die Hyperketonämie erst nach dem 14. Laktationstag erstmals auf. Im Median trat das erste positive Testergebnis in Laktationswoche 2.0 auf (Interquartilsabstand (**IQR**) 1.0 bis 3.5). Von allen an Hyperketonämie erkrankten Tieren wiesen 46.3% nur ein einziges positives Testergebnis während des gesamten Untersuchungszeitraumes auf. Die Häufigkeit positiver Testergebnisse bei erkrankten Tieren lag im Median bei 2 (IQR 1 bis 3). Die längste zusammenhängende hyperketonämische Phase dauerte bei erkrankten Tieren im Median ein Untersuchungsintervall und somit 3 bis 4 Tage an (IQR 1 bis 2 Untersuchungsintervalle). Die Testcharakteristika der 4 untersuchten Testscenarien schwankten abhängig von der Untersuchungsfrequenz und dem verwendeten Goldstandard. Eine einzelne BHB-Messung während des gesamten 42-tägigen Untersuchungszeitraumes erreichte eine Sensitivität von 21% und eine Spezifität von 100% bei Verwendung des Goldstandards G1. Im Gegensatz dazu erreichte ein aus wöchentlichen Messungen bestehendes Testscenario eine Sensitivität von 72% und eine Spezifität von 100% bei Verwendung desselben Goldstandards. Eine

Erhöhung des Goldstandards auf eine Mindestanzahl von 2 positiven BHB-Testergebnissen (G2) ergab für eine einzelne BHB-Messung während des gesamten Untersuchungszeitraumes eine Sensitivität von 33% und eine Spezifität von 97%. Wöchentliche BHB-Messungen erreichten hier eine Sensitivität von 91% sowie eine Spezifität von 83%. Die Ergebnisse der Studie zeigen, dass Hyperketonämie bei Milchkühen mindestens während der ersten 42 Laktationstage auftritt. Dies sollte bei der Planung strategischer Untersuchungsprotokolle berücksichtigt werden. Ein aus 3 BHB-Messungen in zweiwöchigen Abständen während der ersten 42 Laktationstage bestehendes Protokoll erzielt eine Sensitivität von 69% und eine Spezifität von 91% unter Verwendung des Goldstandards G2. Weitere Untersuchungen sind notwendig, um die Effekte einzelner positiver Testergebnisse während des gesamten Untersuchungszeitraumes auf Milch- und Reproduktionsleistung sowie das Risiko der Kühe, assoziierte Erkrankungen zu entwickeln, zu ermitteln.

Ziel der vierten Studie war die Evaluation eines zu dem Zeitpunkt neu im Handel befindlichen elektronischen BHB-Handmessgerätes zur Diagnose der Hyperketonämie bei Milchkühen. Hierzu wurden Blutproben von 155 laktierenden Milchkühen mittels des Gerätes (NovaVet, Nova Biomedical, Waltham, MA, USA) auf ihre BHB-Konzentration hin untersucht. Anschließend wurden die Proben zentrifugiert und die BHB-Konzentrationen der gewonnenen Serumproben in einem kommerziellen Labor gemessen. Die vom Labor ermittelten Werte wurden nachfolgend im Vergleich der beiden Verfahren als Goldstandard eingesetzt.

Die vom Gerät und im Labor gemessenen Werte waren hoch korreliert ($r_s = 0,87$; $P < 0,05$). Zwischen den mit dem Gerät (1,0 mmol/L; IQR 0,7 bis 1,3 mmol/L) und dem Labor (0,9 mmol/L; IQR 0,7 bis 1,1 mmol/L) gemessenen medianen BHB-Konzentrationen wurde eine Differenz von 0,0 mmol/L (IQR -0,1 bis 0,2 mmol/L; $P < 0,05$) festgestellt. Sensitivität und Spezifität des Gerätes betrugen 97% und 82% bei Verwendung eines Grenzwertes von 1,2 mmol/L. Bei Verwendung einer im Labor gemessenen BHB-Konzentration von 1,4 mmol/L als Goldstandard ergab die durchgeführte Receiver-Operating-Characteristic-Analyse die besten Testcharakteristika bei der Verwendung eines optimierten Grenzwertes von 1,3 mmol/L für das Gerät. Sensitivität und Spezifität lagen hierbei bei 96% und 85%. Zusammenfassend kann das Gerät für die Diagnostik der Hyperketonämie beim Milchrind empfohlen werden. Es muss allerdings aufgrund der Spezifität in Höhe von 82% beziehungsweise 85% mit einer gewissen Anzahl falsch als erkrankt diagnostizierter Tiere gerechnet werden.

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9 DECLARATION OF INDEPENDENCE

Hiermit erkläre ich, Annika Mahrt, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Tabelle 1. Eigener Anteil[1] an den Forschungsprojekten der vorliegenden Dissertation

	Studie 1[a]	Studie 2[b]	Studie 3[c]
Studienplanung	+++	+++	+++
Datenerhebung	+++	+++	+++
Datenanalyse	+++	+++	+++
Verfassen des Manuskripts	+++	+++	+++
Editieren des Manuskripts	++	++	++

[1]Legende: +++: >70%
++: 50–70%
+: <50%

[a] Effects of time and sampling location on concentrations of ß-hydroxybutyric acid in dairy cows

[b] Evaluation of hyperketonemia risk period and screening protocols for early-lactation dairy cows

[c] Evaluation eines neuen elektronischen Handmessgeräts zur Messung von ß-Hydroxybutyrat bei Milchkühen

Gut Panker, d. 24.03.2015,

Annika Mahrt