P-ISSN: 2304-3075; E-ISSN: 2305-4360

International Journal of Veterinary Science

www.ijvets.com; editor@ijvets.com

Research Article https://doi.org/10.47278/journal.ijvs/2024.220

A new Assessment Method for the Diagnostic Accuracy of Blood Glucose Analysers for the Diagnosis of Hypoglycemia and Hyperglycemia in Dairy Cows

Abdülkerim Deniz [1,*], Aytaç Pekmezci [2], Kemal Aksoy [3] and Mert Metin [4]

[1]Free Researcher for Clinical Biochemistry, Nisbetiye Mah. 34340 Beşiktaş, İstanbul/Türkiye
[2]University of Muğla Sıtkı Koçman, Faculty of Science, Department of Statistics, 48000 Kötekli, Muğla/ Türkiye
[3]University of Muğla Sıtkı Koçman, Faculty of Veterinary Medicine Milas, Department of Internal Medicine, 48200 Milas, Muğla/Türkiye
[4]University of Muğla Sıtkı Koçman, Faculty of Veterinary Medicine Milas, Department of Biochemistry, 48200 Milas, Muğla/Türkiye
***Corresponding author:** ad.deniz68@gmail.com

Article History: 24-539 Received: 19-Jun-24 Revised: 23-Jul-24 Accepted: 04-Aug-24 Online First: 11-Aug-24

ABSTRACT

The aim of the study was to evaluate the diagnostic accuracy of Gem Premier 3000 (GEM) and Edan i15 Vet (EDAN) for hypoglycemia and hyperglycemia by the analysis of blood glucose concentration (BGC) compared to the reference method (RM) with conventional statistics and using a new method (reversed multiple cut-off points: RMC). BGC was analysed with whole blood at GEM, EDAN and serum at RM in 123 Holsteins in different stages of lactation (DSL). Increasing cut-off points (ICP) (BGC ≥40, ≥45, ≥50, ≥55, ≥60 and ≥65mg/dL) and decreasing cut-off points (DCP) (BGC <40, <45, <50, <55, <60 and <65mg/dL) were defined for the RMC method. Excellent to low limits of agreement with RM (±1 to ±15% deviations) were determined for BGC analysis, differences of absolute and true positive prevalence (APP, TPP) and area under the curve (AUC). Passing-Bablok regression and Bland-Altman plots showed inconsistencies and large deviations for GEM/EDAN in DSL. Sensitivities and specificities were low and different for GEM/EDAN in hypoglycemia/hyperglycemia in receiver operating characteristic. RMC showed that APP and AUC differences were outside the limits for DCP and ICP for GEM and EDAN, also for DSL. The differences in BGC and TPP were outside the limits in DCP for GEM and in DCP and ICP for EDAN. To summarise, BGC in DSL influenced the results of conventional statistics. GEM/EDAN were not accurate for BGC analysis in Holsteins according to conventional statistics, which was also confirmed by the new RMC method in high and low BGC and in DSL. A higher probability of false-identification of hypoglycemia by EDAN and hyperglycemia by GEM according to RMC was observed.

Key words: Diagnostic Accuracy, Edan, Gem, Blood Glucose, Holstein, RMC.

INTRODUCTION

Blood glucose concentration (BGC) must be maintained within a physiological range in all higher organisms, as it is an extremely useful source of energy for the very important organs 'erythrocytes, brain and mammary gland' (Aschenbach et al. 2010). BGC is useful to monitor available energy status along with blood concentrations of non-esterified fatty acids and beta-hydroxybutyrate in lactating Holsteins (Deniz et al. 2020). The onset of lactation requires a massive glucose supply to the mammary gland in Holsteins (Baumgard et al. 2017). This leads to low BGC during early lactation, which triggers mobilisation from lipid depots and increases beta-hydroxybutyrate levels, which can lead to ketosis (Deniz et al. 2020) and reproductive problems associated with low BGC (Oikonomou et al. 2008). Ruminants can suffer from insulin resistance at the end of gestation and the beginning of lactation (Hayirli et al. 2006). BGC is usually monitored by wet biochemical analysis of blood plasma (Mair et al. 2016) or serum in lactating Holsteins (Wittrock et al. 2013; Macmillan et al. 2017). The wet biochemical method of BGC is a reference method (RM) based on a hexokinase reaction (Mair et al. 2016). In addition, portable and easy-to-use electronic devices have been used for rapid analysis of BGC with a drop of blood in dairy cows, but they should be accurate compared to the reference method (Wittrock et al. 2013;

Cite This Article as: Deniz A, Pekmezci A, Aksoy K and Metin M, 2025. A new assessment method for the diagnostic accuracy of blood glucose analysers for the diagnosis of hypoglycemia and hyperglycemia in dairy cows. International Journal of Veterinary Science 14(1): 63-74. https://doi.org/10.47278/journal.ijvs/2024.220

Mair et al. 2016; Macmillan et al. 2017). Point-of-care tests with blood gas devices also provide simple and rapid results in humans (Steinfelder et al. 2006; Vukelic et al. 2007) and also in animals (Deniz et al. 2023; Metin et al. 2023). The accuracy and diagnostic performance of the Gem Premier 3000 (GEM) and Edan i15 Vet (EDAN) blood gas analysers have not been validated for BGC analysis in dairy cows, although GEM and EDAN (Deniz et al. 2023; Metin et al. 2023) are used on the market. It is important to know how accurate they are at low and high BGC as most analysers are produced for humans. A high significant linear correlation between a validated standard method and a test device does not always give a good agreement (Bland and Altman 1986; Zulle 2011; Wittrock et al. 2013; Metin et al. 2023). Therefore, appropriate statistical methods such as the Passing-Bablok regression equation (Jensen and Kjelgaard-Hansen 2006; Zulle 2011; Metin et al. 2023) and Bland-Altman agreement plots (Bland and Altman, 1986; Jensen and Kjelgaard-Hansen 2006; Giavarina 2015; Deniz et al. 2023; Metin et al. 2023) have been used to assess the agreement between a RM and a test device. The acceptable biological variation between subjects and the desirable specification for the total allowable error in biochemical parameters were given by Westgard (2024) and are used for the final decision in the assessment of differences between instruments. However, even with small mean differences (1.4-3.0%) and nearly overlapping regression lines, systematic errors were shown in Passing-Bablok regressions and high total errors in Bland-Altman plots, although high correlation coefficient were observed (Metin et al. 2023). On the other hand, test instruments can show good diagnostic performance despite a disagreement between two methods (Neves et al. 2018; Deniz et al. 2023). These above-mentioned most widely accepted method comparison statistics ultimately indicate whether the test analyser can be used interchangeably with the reference instrument (Jensen and Kjelgaard-Hansen 2006). Bland-Altman plots of agreement do not test instruments at low or high concentrations of biologically variable parameters such as BGC.

There is a need to develop a pragmatic evaluation method for the comparison of blood glucose analysers to test their diagnostic performance at low and high concentrations, especially for the diagnosis of hyperglycemia and hypoglycemia at different stages of lactation (DSL). The aim of the present study was to evaluate the accuracy and diagnostic performance of GEM and EDAN for the analysis of BGC and for the diagnosis of hypoglycemia and hyperglycemia in lactating Holsteins using known statistical methods compared to RM as well as a new assessment method by performing an analysis with multiple cut-off points in the opposite direction corresponding to a low and a high BGC.

MATERIALS AND METHODS

Animal Ethics

This study protocol was approved by the animal ethics committee of Muğla Sıtkı Koçman University (MUDEM-HADYEK) with the approval number of 23.09.2021-31/21.

Study animals and groups

One hundred and twenty-three lactating Holstein cows (n=35 primiparous, n=88 multiparous) were randomly enrolled at calving time and after calving within one month from different dairy farms. The cows were classified according to the time of blood collection in postpartum days (PPD): Calving day (PPD0, n=29), PPD1-3 (n=56), PPD4-9 (n=20) and PPD10-30 (n=18). These groups were formed to observe blood glucose fluctuations and to calculate post-calving hypoglycemia and hyperglycemia rates. Based on the information received from the farms, the cows were fed according to the requirements of their lactation stages (dry, just before lactation, early lactation) and they received water add-libitum.

Blood collection and analysis

Whole blood was collected from a coccygeal vein in a sterile blood collection tube (BD Vacutainer, Becton, Dickinson and company, UK) without anticoagulant. Two mL of whole blood was drawn from the tube into the 100µL lithium heparin-containing injectors (ARD blood gas injector, ADR group) for use in two different blood gas analysers. The lithium-heparinised whole blood was used in GEM (Instrumentation Laboratory Inc. Lexington MA, USA) and EDAN (Edan Instruments, Inc. Shenzhen, China) for whole blood glucose concentration (WBGC) analysis. Approximately 30minutes after the collection of coccygeal whole blood, the blood samples were cool centrifuged without anticoagulant at 4,100rpm for 15minutes (NUVE NF 800R, Nueve San. Mal.). The supernatant serum was removed from the tubes and placed in Eppendorf sample tubes and stored at -20°C for one month until the serum glucose concentration (SGC) was analysed using a commercial test kit in an automated wet biochemical analyser (Mindray BS 120 Vet: Shenzhen Mindray Animal Medical Technology Co., LTD). SGC analysis was based on the hexokinase reaction, and it was a RM for comparison with EDAN and GEM. GEM and EDAN used the amperometric method (reaction with oxygen in the presence of glucose oxidase: hydrogen peroxide determination in the platinum electrode) to analyse WBGC. Samples were used within 5minutes of collection and mixed at room temperature by gentle manual rotation without using an ice block as recommended by the manufacturer. The reportable range for WBGC was 0.00-999 and 20.0-700mg/dL for GEM and EDAN, respectively. Calibration of Mindray BS 120 Vet (BS120), EDAN and GEM was performed in advance using the respective manufacturer's reference kits before starting the analysis of the samples.

Statistical analysis

The software MedCalc (MedCalc Software Ltd, Belgium) version 2022 was used to perform the statistical analyses. The normality of the data was checked using the Schapiro-Wilk test. Where necessary, descriptive statistics were presented as x±SD (mean and standard deviation). The mean BGCs of RM, EDAN and GEM were compared using the Wilcoxon test in DSL. The significance level for all statistical tests was set at α=0.05. Least squares regression analysis (LSRA) was used for the correlation coefficients and 95% confidence intervals of the WBGC

and SGC analysed by GEM/EDAN and RM. Bland-Altman plots of agreement were performed for bias between quantitative results from three instruments (Bland and Altman 1986; Giavarina 2015). Method comparison using Passing and Bablok regression equation (Jensen and Kjelgaard-Hansen 2006; Zulle 2011) was performed to observe the agreement between GEM and RM (GEM/RM) and between EDAN and RM (EDAN/RM). Receiver operating characteristics (ROC) analyses (Fawcett 2006; Simundić 2009) were performed to determine the area under the curve (AUC), specificity (Sp) and sensitivity (Se) for the diagnosis of hypoglycemia and hyperglycemia. In addition, the number of true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN) detected by EDAN and GEM based on RM was calculated to define diagnostic performance (Fawcett 2006; Simundić 2009; Baratloo et al. 2015; Trevethan 2017). True positive prevalence (TPP) based on TP by RM and absolute positive prevalence (APP, independent of RM) of hypoglycemia and hyperglycemia at calving and postpartum were calculated as percentages.

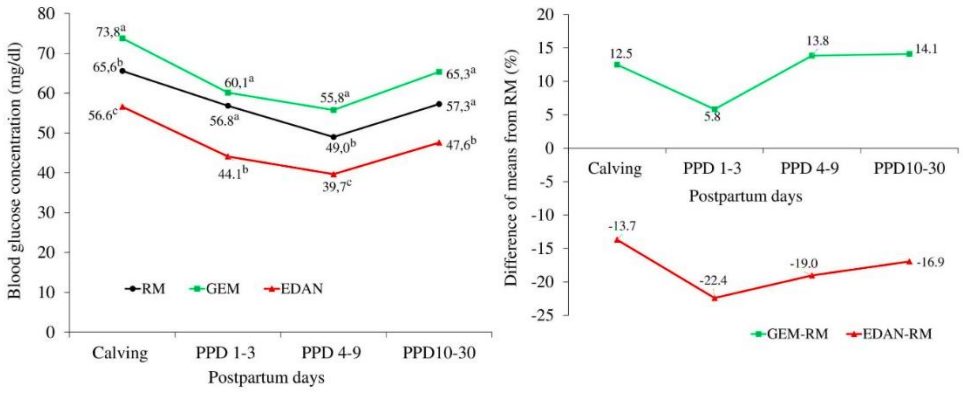
Description of reverse multiple cut-off points (RMC) method

The RMC method was developed for the diagnostic accuracy of test devices compared to a RM to observe the response of test devices at low and high BGCs and at DSL. As a first step, a series of decreasing cut-off points (DCP) and increasing cut-off points (ICP) were established for the RMC method based on a physiological reference range for BGC in Holsteins. Thus, RMC tested the diagnostic performance of the test devices within and outside the physiological normal range for DCP and ICP. The first physiological normal range for BGC was 40-60mg/dL in lactating Holsteins (Mair et al. 2016). Some studies recommend a BGC of <2.5mmol/L (<45.5mg/dL) to detect hypoglycemia (Wittrock et al. 2013). Therefore, 45-65mg/dL was also used for BGC as a second normal range in lactating Holsteins. Thus, BGC <40 and <45mg/dL for hypoglycemia and BGC ≥60 and ≥65mg/dL for the definition of hyperglycemia were set as cut-off points. In addition, the ranges for DCP were defined as 'BGC <40, <45, <50, <55, <60 and <65mg/dL' and for ICP as 'BGC ≥40, ≥45, ≥50, ≥55, ≥60 and ≥65mg/dL' to evaluate the diagnostic performance of testing devices using the RMC method. At each cut-off point, a unit of 5mg/dL was chosen for increased or decreased BGC compared to the next cut-off point in a reverse trend to test the diagnostic performance at low and high BGC. This unit could be 1, 2 or 3mg/dL for each cut-off point, which intensifies the analysis. In a second step, the mean of BGC in DSL for RM/GEM/EDAN was calculated as mean±SD and the coefficients of variation (CV(%)=100xstandard deviation/mean) were presented. The population mean of the BGC at each cut-off point was calculated separately for each test device as descriptive statistics. This calculation was performed for the cases TP by RM, APP and TPP by EDAN and GEM. In a third step, the percentage difference between the BGC mean values of GEM/EDAN and RM in DCP and ICP was calculated. This shows the BGC variations at low and high concentrations and the differences of GEM and EDAN to RM in TP by RM, APP and TPP. In a fourth step, the number of values that were within or outside DCP and ICP was counted and their ratio to the population was calculated in % to show APP and TPP, also in DSL for hypoglycemia and hyperglycemia. In addition, the AUC for RM, EDAN and GEM at DCP and ICP was calculated based on the formula for the respective trapezoids [AUC=1/2(a+b)h] in RMC. In a fifth step, the differences of APP and TPP between GEM/RM and between EDAN/RM at each DCP and ICP relative to RM were calculated (assuming 100% coverage of the values at each DCP and ICP by RM). This difference was presented in the total population and in DSL for hypoglycemia and hyperglycemia. In a sixth step, the acceptance limits for the difference of GEM/RM and EDAN/RM of APP and TPP and for the average BGCs in DCP and ICP were determined considering the literature reports and the clinical aspect of BGC variation in DSL in dairy cows. The acceptable limits for GEM/RM and EDAN/RM were set as follows: between ±1.0% and 0.0% (excellent), ±2.5% and ±1.0% (fairly good), ±2.5% and ±5% (good), ±5% and ±10% (moderate), ±10% and ±15% (low) and >15% (unacceptable) for DCP and ICP. Westgard (2024) gave a desirable specification for an acceptable overall error of 5.5-6.96% for plasma and serum BGC from RM. In addition, according to the American Society for Veterinary Clinical Pathology guidelines (Gerber and Freeman 2016), individual glucose results from the glucose analyzer should be within 10% of the reference value for values below the reference interval and within 20% of the reference value for values within and above the reference interval.

RESULTS

Results of conventional statistic

The mean, minimum and maximum BGC of the study animals were 63.39±19.09 (29-178), 46.82±17.67 (18-144) and 57.67±18.36 (21-153)mg/dL for GEM, EDAN and RM, respectively (P<0.05). Mean BGC between methods differed significantly on calving day and other postpartum days (P<0.05), except on PPD1-3 between GEM/RM (Fig. 1). BGC decreased significantly from calving to PPD4-9 within the device groups (P<0.05). There was no significant difference between PPD10-30 and other postpartum days within the device groups. The CV changed significantly within the device groups between calving and PPD30 (Fig. 1). LSRA and Passing-Bablok regressions in DSL were shown in Fig. 2. LSRA showed that there was a significant positive correlation for GEM/RM and EDAN/RM. There was agreement between GEM/RM excluding lactation stage in PPD0-30, showing that intercept A included 0.0 (95% CI: -4.606 to 10.544) and slope B 1.0 (95% CI: 0.886 to 1.162). However, the Passing-Bablok regression equation showed no agreement between EDAN/RM (95% CI of A: -16.479 to -0.020, B: 0.777 to 1.066), when excluding lactation stages in PPD0-30, there was a constant error. The Passing-Bablok regression equation differed between BGCs, especially in PPD4-9 and PPD10-30, and there was no agreement between GEM/RM and EDAN/RM. Agreement was observed between EDAN/RM (95% CI of intercept A: -23.044 to 7.079; slope B: 0.739 to 1.169) and between



Standard deviation of BGC (± mg/dl)

	Calving	PPD1-3	PPD4-9	PPD10-30
RM	27.34	13.04	14.31	14.48
GEM	28.75	12.50	18.63	8.27
EDAN	26.12	12.71	15.20	9.31

Coefficient of variation of BGC (%)

	Calving	PPD1-3	PPD4-9	PPD10-30
RM	41.70	22.95	29.22	25.29
GEM	38.98	20.79	33.42	12.66
EDAN	39.83	28.83	38.34	19.58

Fig. 1: Mean blood glucose concentrations using GEM and EDAN blood gas analysers (in whole blood) and reference method (RM: BS120 wet biochemistry analyser in serum) in 123 lactating Holstein cows at calving, postpartum days (PPD) 1-3, PPD4-9 and PPD10-30 and percentage difference in mean values of GEM and EDAN compared to RM. BGC: Blood glucose concentration. BGC significantly decreased between calving and PPD1-3, PPD 4-9 ($P<0.05$), but not at PPD10-30 ($P>0.05$) within each device group. [a, b, c]: different letters on the respective postpartum days indicate significant differences between RM, EDAN and GEM ($P<0.05$).

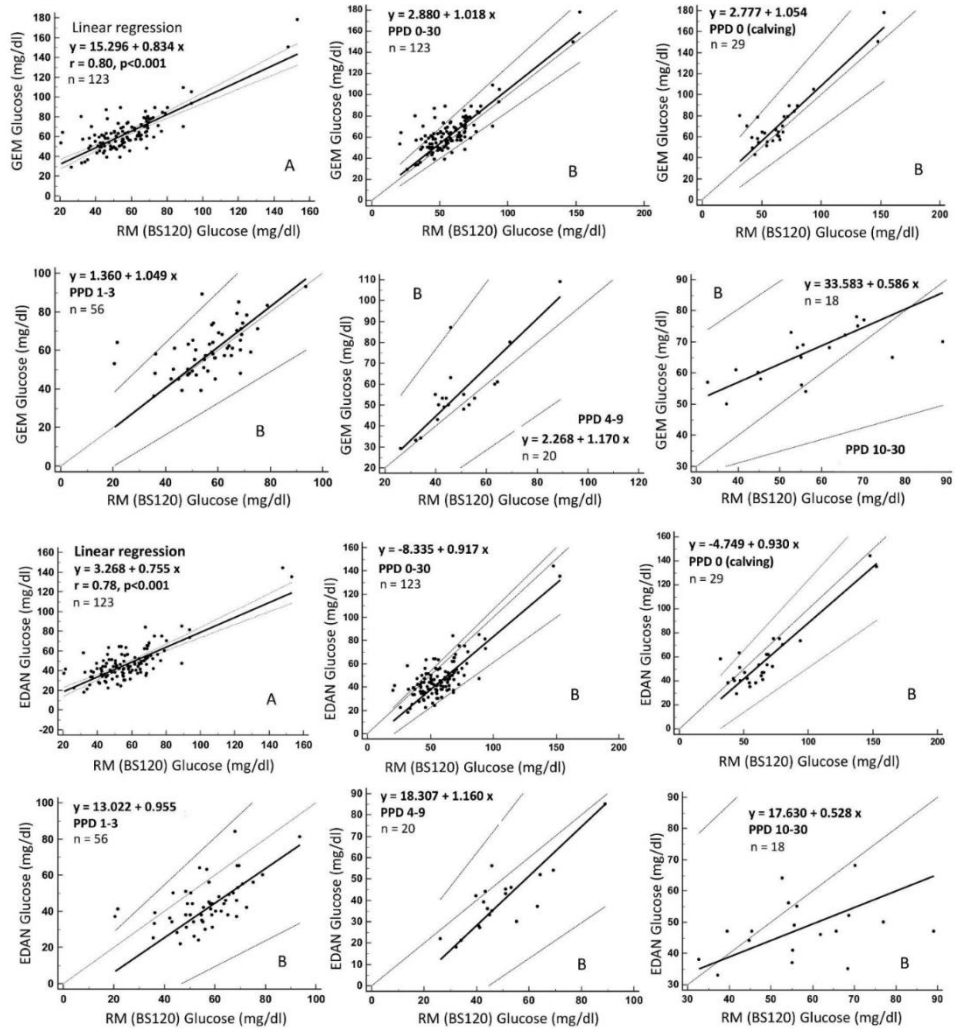


Fig. 2: Linear regression analysis (A) and Passing-Bablok regression equation (B) with 95 % confidence intervals (CI) for the analysis of blood glucose concentration between the analysers GEM Premier 3000 (GEM) and EDAN i15 Vet (EDAN) and the reference method (RM) (Mindray BS120, wet biochemistry) in 123 lactating Holstein. PPD0-30: Cows between calving and postpartum day 30 (PPD30). PPD0 (calving): Cows on the day of calving. PPD1-3: Cows between day 1 and 3 postpartum. PPD4-9: Cows between day 4 and 9 postpartum. PPD10-30: Cows between days 10 and 30 postpartum. 45º line (Passing-Bablok): Identity of RM. Grey lines: 95% CI. Dark lines indicate agreement or error of the respective devices in the Passing-Bablok regression.

GEM/RM (intercept A 95 CI: -14.331 to 19.043; slope B 95% CI: 0.829 to 1.288) at calving day in the Passing-Bablok regression equation, but the intercept error looked very high. Bland-Altman plots (Fig. 3) showed a mean deviation of -10.9mg/dL (-18.8%) for EDAN/RM, 5.7mg/dL (9.9%) for GEM/RM. The total deviations were 46.3mg/dL and 46.5mg/dL for GEM/RM and EDAN/RM, respectively. The mean and total bias changed as a function of BGC variation at DSL. The total bias increased at calving to 50.2mg/dL for the GEM/RM agreement plots. For the EDAN/RM agreement plots, it was 57.3mg/dL at PPD10-30. The Se, Sp and AUC provided by the ROC analysis were shown in Table 1. The ROC analysis showed that the calculated total (the SUM)

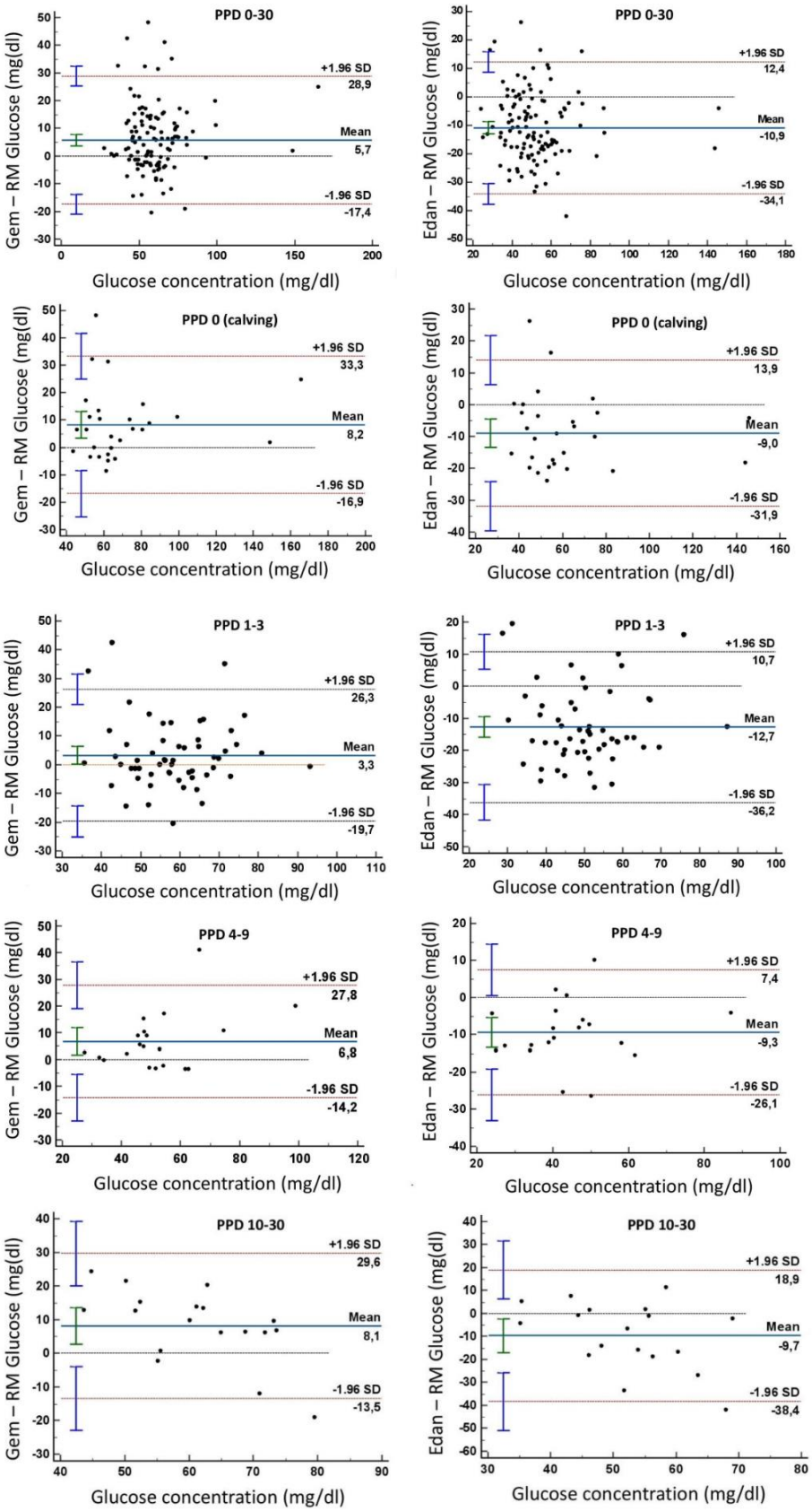


Fig. 3: Bland-Altman plots of agreement in venous blood glucose concentration analysis between the GEM Premier 3000 (GEM) and EDAN i15 Vet (EDAN) blood gas analysers and the reference method (RM) (Mindray BS120, wet biochemistry) in 123 lactating Holstein. PPD 0-30: Cows between calving and postpartum day 30. PPD 0 (calving): Cows on the day of calving. PPD4-9: Cows between day 4 and 9 postpartum. PPD1-3: Cows between days 1-3 postpartum. PPD10-30: Cows between days 10 and 30 postpartum. The dashed line, which adjusts to the value zero, indicates the zero bias. Dark line (mean): shows the bias of the respective unit compared to RM. Dashed lines: show the upper and lower limits of agreement (±1.96 standard deviation, SD).

of sensitivity and specificity for detection of hypoglycemia by EDAN and GEM were 142 and 126.7% at SGC<40mg/dL, respectively, and 121 and 145% at SGC<45mg/dL for EDAN and GEM, respectively. Similarly, the SUM did not exceed 180% for the detection of hyperglycemia for both test devices (Table 1). The AUC by ROC was higher in hypoglycemia detection for EDAN, in contrast it was higher in hyperglycemia detection for GEM. An increasing BGC led to a decreased Se of EDAN, conversely a decreasing BGC led to a decreased Se of GEM. Due to the FP rates of EDAN and GEM, the APP were higher for the detection of hypoglycemia and hyperglycemia, respectively (Table 1).

Table 1: Receiver Operating Characteristics (ROC) analysis for the detection of hypoglycemia and hyperglycemia by GEM and EDAN based on the values of the reference method (hypoglycemia: serum glucose concentration <40 or <45mg/dL, hyperglycemia: serum glucose concentration ≥60 or ≥65mg/dL) in 123 lactating Holsteins

Cut-off points of SGC/WBGC and test devices		ROC analysis					Prevalence of hypo- and hyperglycemia (%)	
		Sp (%)	Se (%)	AUC	*j*	P value	TPP*	APP**,[1]
<40mg/dL	RM	100.0	100.0	100.0	1.00	<0.001	11.4	11.4
	GEM	98.2	28.6	0.63	0.27	=0.034	3.3	4.9
	EDAN	70.6	71.4	0.71	0.42	=0.002	8.1	34.1
<45mg/dL	RM	100.0	100.0	100.0	1.00	<0.001	21.1	21.1
	GEM	97.9	23.1	0.61	0.21	=0.014	4.9	6.5
	EDAN	56.7	88.5	0.73	0.45	<0.001	18.7	52.8
≥60mg/dL	RM	100.0	100.0	100.0	1.00	<0.001	39.0	39.0
	GEM	70.7	89.6	0.80	0.60	<0.001	35.0	53.7
	EDAN	94.7	31.3	0.63	0.26	<0.001	12.2	15.4
≥65mg/dL	RM	100.0	100.0	100.0	1.00	<0.001	29.3	29.3
	GEM	81.6	77.8	0.80	0.59	<0.001	22.8	35.8
	EDAN	100.0	33.3	0.67	0.33	<0.001	9.8	9.8

RM: Reference Method (BS120 wet biochemistry). TPP: True positive prevalence (based on the cut-off points by RM). APP: Absolute positive prevalence for EDAN and GEM (includes true positive, false positive and negative). AUC: Area under the curve. *j*: Youden Index. Se: Sensitivity. Sp: Specificity. EDAN: Edan i15 Vet blood gas device. GEM: Gem Premier 3000 blood gas device. SGC: Serum glucose concentration by RM. WBGC: whole blood glucose concentration by GEM and EDAN. *: Based on SGC by RM. **: Based on WBGC by EDAN and GEM, not based on RM. [1]: APP does not refer to ROC analysis, rather it refers to reverse multiple cut-off point analysis.

Table 2: Number of true-positive cases with the reference method (RM), absolute positive prevalence (APP) and true-positive prevalence (TPP) with EDAN and GEM at decreasing cut-off points (DCP) and increasing cut-off points (ICP) of the blood glucose concentrations (BGC) analysed with the blood gas analysers GEM, EDAN and RM in 123 lactating Holsteins.

BGC cut-off points	TP by RM (n^1)	APP (n^2)		TPP (n^3)	
(mg/dL)	RM/EDAN/GEM	GEM	EDAN	GEM	EDAN
<40	14	6	42	4	10
<45	26	8	65	6	23
<50	39	21	83	14	33
<55	55	35	97	30	49
<60	75	57	104	52	71
<65	87	79	111	71	87
≥40	109	117	81	107	77
≥45	97	115	58	95	55
≥50	84	102	40	77	34
≥55	68	88	26	63	20
≥60	48	66	19	43	15
≥65	36	44	12	28	12
>40<60	62	51	58	40	31
>45<65	61	68	44	43	22

n^1: Number of true positive (TP) cases detected by RM and applied to EDAN and GEM (includes TP, false positive and negative cases in GEM and EDAN). n^2: Number of cases for APP detected by GEM and EDAN independently from RM (includes TP, true negative, false positive and false negative). n^3: Number of correct cases for TPP detected by GEM and EDAN based on TP cases of RM (includes TP only).

Results of RMC analysis

The number of cases for TP by RM, APP and TPP by GEM and EDAN were presented in Table 2. The average BGCs and BGC difference of GEM/RM and EDAN/RM were shown in Fig. 4. The BGC difference of GEM/RM based on TP cases by RM was positive and within acceptable limits at ICP and was categorised as fair to good agreement, but not at DCP. The BGC difference of EDAN/RM based on TP cases by RM was within acceptable limits between <40 and <55mg/dL only at DCP. All BGC differences of EDAN from RM were not within acceptable limits for ICP. All BGC differences of GEM/RM based on APP for GEM were within acceptable limits at DCP and ICP and were categorised as moderate agreement. For EDAN, large deviations in the BGC difference of RM were observed for ICP and DCP, especially at the cut-off point ≥50mg/dL. The agreement was low. Most of the BGC differences of GEM/RM based on TPP were within the acceptable limits, which were classified as excellent to moderate for DCP and ICP. Large variations in the BGC differences of EDAN/RM based on TPP were observed in DCP and ICP, especially at the cut-off point of 50mg/dL. The agreement was low and unacceptable. Fig. 5 shows the APP and TPP of EDAN and GEM in the total population in DCP and ICP. The difference in hypoglycemia detection between EDAN/RM was 200 and 150% for BGC<40 and <45mg/dL, respectively. The difference for the detection of hyperglycemia was -60.4 and -66.7% for BGC≥60 and 65mg/dL respectively. The difference for EDAN/RM was not within acceptable limits for either DCP or ICP. This indicates no diagnostic agreement with RM in DCP and ICP. The differences between GEM/RM were -57.1 and -69.7% for hypoglycemia findings and 37.5 and 22.2% for hyperglycemia findings. There was moderate diagnostic agreement with RM only for the cut-off point of BGC ≥40mg/dL, but no further agreement was observed at other ICP cut-off points. This indicates no diagnostic

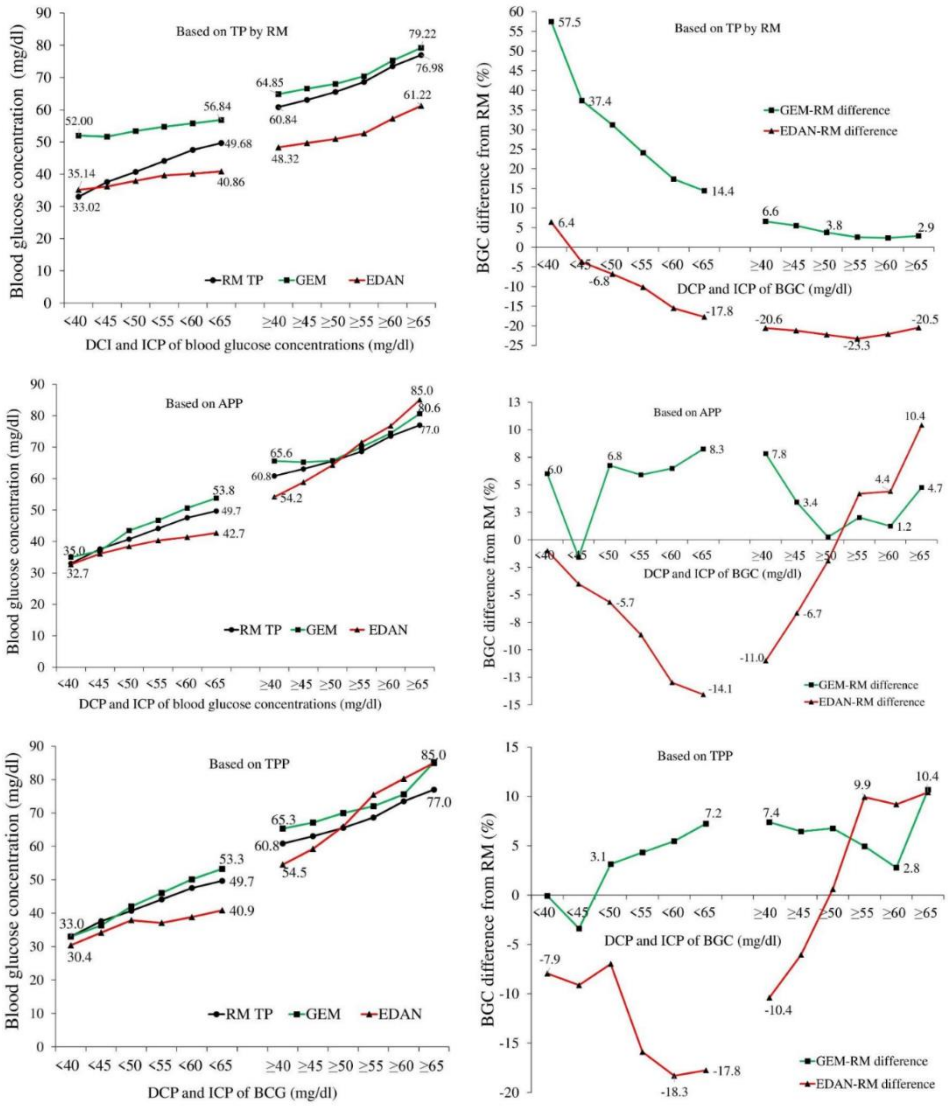


Fig. 4: Reverse multiple cut-off point analysis for serum and whole blood glucose concentrations (BGC) and the percentage difference from the reference method (RM) at decreasing cut-off points (DCP) and increasing cut-off points (ICP) for the EDAN and GEM blood gas analysers in 123 lactating Holsteins. TP by RM: True positive cases detected by RM, average BGC of all devices were calculated to the same numbers of TP by RM (see Table 2). APP: Absolute positive prevalence cases by EDAN and GEM (including false positive and negative cases) (see Table 2). TPP: Correct true positive prevalence cases detected by EDAN and GEM based on TP by RM (see Table 2).

Table 3: Prevalence of hypoglycemia (BGC<45mg/dL) and hyperglycemia (BGC≥65mg/dL) calculated using true positive prevalence (TPP) and absolute positive prevalence (APP) and differences from the reference method (RM) at calving, postpartum days 1-3 (PPD1-3), 4-9 (PPD4-9) and 10-30 (PPD10-30), determined using the reference method, GEM Premier 3000 (GEM) and EDAN i15 Vet (EDAN) in lactating Holstein cows.

BGC	Device	TPP/APP	Prevalence and difference from RM (%)			
			Calving	PPD1-3	PPD4-9	PPD10-30
<45mg/dL	RM	TPP	17.2	14.3	45.0	22.2
	GEM	TPP	3.4	1.8	20.0	0.0
		Difference from RM	-80.0	-87.5	-55.6	-100.0
		APP	3.4	5.4	20.0	0.0
		Difference from RM	-80.0	-62.5	-55.6	-100.0
	EDAN	TPP	13.8	10.7	45.0	16.7
		Difference from RM	-20.0	-25.0	0.0	-25.0
		APP	37.9	60.7	70.0	33.3
		Difference from RM	120.0	325.0	55.6	50.0
≥65mg/dL	RM	TPP	41.4	28.6	10.0	33.3
	GEM	TPP	31.0	23.2	10.0	33.3
		Difference from RM	-25.0	-18.8	0.0	0.0
		APP	44.8	30.4	15.0	61.1
		Difference from RM	8.3	6.3	50.0	83.3
	EDAN	TPP	20.7	7.1	5.0	5.6
		Difference from RM	-50.0	-75.0	-50.0	-83.3
		APP	20.7	7.1	5.0	5.6
		Difference from RM	-50.0	-75.0	-50.0	-83.3

BGC: Blood glucose concentration. RM: Reference method (wet biochemistry according to BS120) was accepted with a 100% correct detection of prevalence for the calculation of the true positive prevalence. TPP: Samples detected by RM at the respective cut-off points for hypoglycemia and hyperglycemia, as well as the same samples detected by GEM and EDAN. APP: Samples recognized by GEM and EDAN independently of RM.

agreement with RM at ICP and DCP. The AUC differences of EDAN and GEM confirmed that GEM in ICP and EDAN in DCP had a much lower difference with RM (better sensitivity) for TPP, but this result was due to the high FN/FP detection by EDAN in DCP and by GEM in ICP, as shown by the AUC differences for APP (Fig. 5). There was no agreement in the detection of TP cases in DCP and ICP for both devices, therefore no diagnostic agreement with RM was observed. RM results showed that the rate of hypoglycemia and hyperglycemia was different on the day of calving and after birth (Table 3). These results were not confirmed by EDAN and GEM, so there were large differences from RM concerning TPP and APP. EDAN and GEM were unable to differentiate the distinct risk of hypoglycemia and hyperglycemia at the different postpartum stages based on FP and FN rates. The difference between GEM/RM for TPP for hyperglycemia and between EDAN/RM for TPP for hypoglycemia detection was consistently zero at PPD4-9 or PPD10-30, but the APP difference did not confirm these results as much higher FP detections were observed. GEM and EDAN measured opposite WBGC at DCP and ICP, even on the different days of postpartum. Both had no diagnostic agreement with RM, even in DSL.

DISCUSSION

The accuracy and correlation with the reference method of various portable rapid test glucometers in blood obtained from the capillary, jugular vein or coccygeal vessel has been reported by others (Voyvoda et al. 2010; Wittrock et al. 2013; Mair et al. 2016; Macmillan et al. 2017; Nakadate et al. 2019). The present study showed a significant positive correlation between GEM/RM and EDAN/RM. Similar correlation coefficients were reported by Mair et al. (2016) for other devices. However, this was not sufficient to make a statement about the agreement and correct diagnostic performance of the test instruments. A significantly high correlation is not fully suitable for assessing the diagnostic performance and agreement of the test instruments (Bland and Altman 1986; Zulle 2011; Wittrock et al. 2013; Metin et al. 2023). Linear regression is based on random errors in the dependent variables. The Passing-Bablok regression equation is a powerful statistical analysis for the agreement of methods that provides information on the proportional and systematic errors by testing 95% confidence intervals for intercept A and slope B (Jensen and Kjelgaard-Hansen 2006; Zulle 2011). Perfect

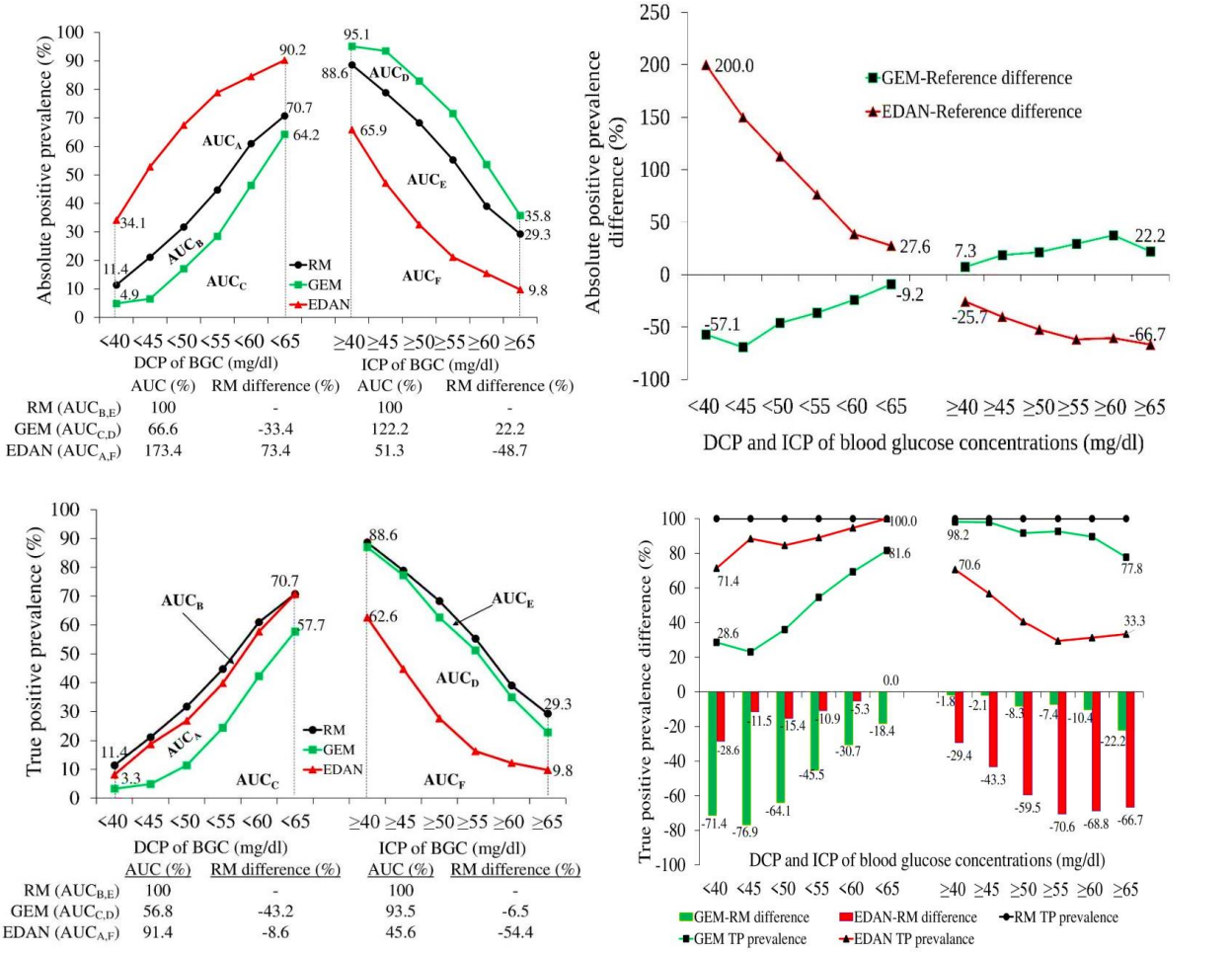


Fig. 5: Reverse multiple cut-off points analysis for absolute and true positive prevalence and their differences from reference method (RM) prevalence for GEM and EDAN in 123 lactating Holsteins with decreasing cut-off points (DCP, <40 to <65mg/dL) and increasing cut-off points (ICP, ≥40 to ≥65mg/dL) of blood glucose concentration (BGC). Absolute positive prevalence: values detected by EDAN and GEM that are within the cut-off point regardless of RM. TP (true positive): correct cases recognized by EDAN and GEM, true positive prevalence according to RM. AUC: Area under the curve. AUC_A: AUC of EDAN at DCP. AUC_B: AUC of RM at DCP. AUC_C: AUC of GEM at DCP. AUC_D: AUC of GEM at ICP. AUC_E: AUC of RM at ICP. AUC_F: AUC of EDAN at ICP.

agreement with no error between the methods shows that the adjusted Passing-Bablok regression masks the 45-degree line (y=x) (Jensen and Kjelgaard-Hansen 2006; Zulle 2011). The Passing-Bablok regression equation in the present study showed agreement between GEM/RM, but not between EDAN/RM independent of DLS for PPD0-30. Despite agreement between GEM/RM in the Passing-Bablok regression, insufficient Se in the ROC analysis was observed in the detection of hypoglycemia and hyperglycemia. However, despite the inconsistencies in the Passing-Bablok regression, the test devices can show good diagnostic performance in the ROC analysis (Mair et al. 2016; Deniz et al. 2023). This is a contradictory situation that needs to be clarified by further statistical analyses. There was no agreement with RM, especially for PPD4-9 and PPD10-30 for both GEM and EDAN, although GEM agreed with RM for the whole population without considering DSL. It is likely that low and high BGC and their biological variation related to the time of sampling after calving have an impact on this analysis in dairy cows. As shown in the present study, the Passing-Bablok regression equations of EDAN and GEM looked completely different for DSL after calving. Although the intercept at calving included 1.0 for both the GEM and EDAN regression equations, the intercept error was too high from a clinical perspective, which is consistent with the high overall error in the Bland-Altman agreement plots. This disagreement was clearly observed in the significant differences between the BGC averages of GEM/RM and EDAN/RM and in the differences between APP and TPP of RM for the detection of hypoglycemia and hyperglycemia in the RMC analysis at calving. If a method comparison with the Passing-Bablok regression equation is to be performed, the sampling time and biological BGC fluctuations after calving must be taken into account. The CV was conspicuously altered in all methods during the transition period in dairy cows, which may be one reason for this. The insufficient Se of EDAN/GEM in ROC analysis was observed for the detection of hypoglycemia and hyperglycemia concerning the whole population that was confirmed by the RMC method in DLS, as TPP and APP difference were out of limits due to the high FP rate and insufficient TP rate. In addition, inconsistent regression equations of EDAN/GEM compared to RM provided by Passing-Bablok on DLS were observed in BGC at DCP and ICP as well as in the difference of BGC to RM generated by the RMC method.

Furthermore, the Bland-Altman plots show the intervals of agreement and bias, but it does not say whether the limits are acceptable or not (Giavarina 2015). The Bland-Altman plots showed total bias was slightly more than 46mg/dL for GEM/RM and EDAN/RM, which was above the acceptable specification (5.5-6.96%) for BGC according to Westgard (2024). The mean and total bias in the Bland-Altman plots of agreement depended on the biological BGC variations in DSL after calving. Especially, a high total bias was observed in PPD10-30, which was not acceptable from a clinical point of view. BGC at DCP and ICP based on TPP and APP were determined at different time points after calving in relation to the RMC method, where BGC can fluctuate considerably depending on the energy requirements of the dairy cows for milk production, which can lead to hypoglycemia or hyperglycemia, as shown by the BGC fluctuation after calving up to PPD30 in the present study. Lactating cows have a high demand for glucose, as the mammary gland needs it for the start of lactation (Baumgard et al. 2017). Therefore, the onset of milk production and negative energy balance in the early lactation phase may be a reason for hypoglycemia in PPD4-9 in the present study. Hayırlı et al (2006) reported insulin resistance in the last stage of gestation and early lactation, which is consistent with the present study, so a higher prevalence of hyperglycemia at calving was observed according to RM. RMC showed that the difference of BGC for GEM at DCP and for EDAN at ICP was totally different and differences according TP by RM were out of the limits. In addition, EDAN showed inconsistency at ICP in terms of BGC fluctuations according APP and TPP compared to RM. Therefore, the timing of blood sampling in dairy cows may have an influence on the average values of BGC and the prevalence of diseases, as shown in the present study. These are not taken into account when analysing with the Bland-Altman Plots. Bland-Altman plots provide a mean and a total error based on 95% CI in a population (Bland and Altman 1986; Zulle 2011; Giavarina 2015). A mean error provided by Bland-Altman is similar to the mean arithmetic difference of BGC between GEM/EDAN and RM. RMC provided mean differences of the BGC from RM/EDAN and RM/GEM based on the TP of RM, APP and TPP. Remarkably, it was observed that the mean BGC differences in DCP and ICP vary among three different options such as TP of RM, APP and TPP of GEM and EDAN. According to the American Society for Veterinary Clinical Pathology guidelines, only 54.6% to 35.1% of glucose readings had an overall observable error ≤20%. Based on these guidelines, the performance of the device can be considered acceptable if the quality control material was analysed on 5 different days and the total error is ≤20% (Harr et al. 2013). However, as the present study has shown, measuring BGC without considering the biological variations in DLS, DCP and ICP is not sufficient to draw conclusions about the agreement and diagnostic perfomance of the test device, as there are large variations in BGC as well as in APP and TPP.

A significant linear correlation provided by LSRA, regression equation by Passing-Bablok and Bland-Altman plots of agreement do not show the correct performance and agreement of analysers with RM if the biological fluctuation of BGC in dairy cows was not taken into account. Conventional test methods need to be applied to the low and high concentrations of BGC in DSLs due to significantly fluctuating BGC and remarkable changed CV. The pattern of hypoglycemia and hyperglycemia were observable both in the significant fluctuations of BGC and in the APP and TPP difference from RM between calving and PPD30 in RMC analysis. RMC method showed the real diagnostic performance of GEM and EDAN compared to RM by showing overall BGC, APP and TPP differences at DCP and ICP including in DSL, which were out of acceptable limits.

On the other side, an AUC of 60-70% and 80-90% was found to be sufficient and very good in the ROC analysis respectively (Simundić 2009). A sufficient AUC

above 60% provided by ROC for GEM and EDAN in hypo- and hyperglycemia detection did not result in sufficient Se and Sp rates. The SUM of Se and Sp did never exceed 180% for hypoglycemia and hyperglycemia diagnosis. The Se of GEM looked better when diagnosed hyperglycemia and much worse when diagnosed hypoglycaemia, and vice versa for EDAN according to ROC analysis. AUC was a measure of the powerful detection of a test to identify animals sick or healthy, in the present study hypoglycemic or hyperglycaemic cows. An AUC of 1.0 showed an excellent test; but if it was below 0.5, it meant a worthless test (Swets 1988; Fawcett 2006; Simundić 2009). Moreover, a sensitivity of 50% and specificity 100% or vice versa indicated an accuracy of 75% of test devices, that meant the device can be used either for the screening the population or for the diagnosis of the disease, but not for both (Baratloo et al. 2015). There is a contradictory situation between AUC and Se/Sp rates for the correct diagnosis of diseases at hypo- or hyper-concentration of the respective parameters. Different cut-off points of the respective parameter for the diagnosis can result in different AUC, Se and Sp in ROC analysis as it was reported by others (Jansen et al. 2021; Deniz et al. 2023) and as shown in the present study. ROC analysis refers TP cases and TN cases based on RM for the determination of Se and Sp (Fawcett 2006; Baratloo et al. 2015; Trevethan, 2017). The APP and TPP difference of GEM/EDAN from RM did not fell within the acceptable limits of RMC analysis at DCP and ICP, even at critical time points after calving for hypoglycemia and hyperglycemia detection. The difference was frequently above 10% and up to 200% at DCP and ICP, and up to 325% at critical times after calving in RMC analysis, although the Se and Sp of GEM were above 70% for hyperglycemia detection calculated by ROC analysis. The Se related to the potential of a test to recognise subjects with the disease and the Sp to recognise subjects healthy and without disease, both of them are expressed in percentage and define the proportion of TP and TN subjects in a total group (Fawcett 2006; Simundić 2009; Trevethan 2017). Although EDAN performed slightly better in detecting hypoglycemia and GEM in detecting hyperglycemia according to ROC, this was only due to high probability because EDAN frequently measured too low WBGC and in contrast, GEM systematically measured too high WBGC. This is the reason why the Se of GEM was high in hyperglycemia and EDAN in hypoglycemia. This can also be observed at AUC differences of EDAN and GEM in APP and TPP in the RMC analysis. The RMC analysis provided detailed information on the diagnostic performance of the two test devices at low and high BGC. For example, the difference in detection of absolute hypoglycemia cases by EDAN and GEM compared to RM at DCP was very high for EDAN and very low for GEM. Controversially, the difference in detection of absolute hyperglycemia cases by EDAN and GEM compared to RM at ICP was very low for EDAN and very high for GEM. This proves that GEM systematically measures high WBGC, but EDAN frequently measures low WBGC, as it was presented in BGC fluctuations between calving and PPD30. The diagnostic accuracy of GEM and EDAN was much worse at DCP and ICP. GEM was not able to find hypoglycemic cases at calving and at PPD10-30. On the contrary, EDAN identified more cases of hypoglycemia in the whole days after birth. Conversely, the rate of hyperglycemia cases identified by GEM was much more than by RM, but not by EDAN. A test with high specificity and sensitivity with low rate of false positive and false negative has high diagnostic odds ratio, so that this test with sensitivity >90% and specificity of 99% has a diagnostic odds ratio greater than 500 (Simundić 2009). That diagnostic odds ratio was far away for GEM and EDAN at DCP and ICP thus the diagnostic accuracy of both devices was very low according to RMC analysis. Although the BGC difference of GEM/RM at ICP showed a good agreement with RM, this was because GEM systematically measured high BGC. BGC difference of GEM in DCP was out of acceptable limits because the device was not sensitive enough for low BGC. Wittrock et al. (2013) used coccygeal whole blood without preservative for BGC analysis by a handheld glucometer compared to RM in dairy Holstein and observed a high correlation coefficient and good agreement in Bland-Altman plots. The Se and Sp were 84 and 52% at the insulin sensitivity test respectively. The agreement and sensitivity were much lower in high BGC induced by glucose infusion rather than in normal physiological range. As shown in the present study, it confirmed that a positive correlation cannot result in high agreement and sensitivity of the diagnostic test devices in high or low BGC. However, Wittrock et al. (2013) recommended using the device despite fluctuations in the sensitivity and agreement at higher BGC, whereas the present study cannot recommend GEM and EDAN because EDAN created high bias at DCP and GEM at ICP, they were even not able to differentiate hypo- and hyperglycemia in DLS according to RMC and ROC. The reason for that was that GEM delivered systematically high, EDAN delivered always low WBGC, thus the probability was high, but incorrect to detect hyperglycemia and hypoglycemia by GEM and EDAN respectively. GEM was tested for the accuracy and precision in human by others (Steinfelder-Visscher et al. 2006; Vukelic et al. 2007; Roeder et al. 1996) and found in good agreement, even it was used as a standard comparison method. But there was no published paper about use of EDAN for BGC analysis in human or in dairy Holstein. The technical user instruction of EDAN and GEM provided information about a high accuracy and linearity with other devices only. However, these results were not applicable for WBGC analysis in dairy Holsteins. EDAN was also validated in comparison to GEM for the analysis of blood ionised calcium (Deniz et al. 2023), however both devices were not to use interchangeably although their diagnostic performance were comparable. The accuracy, sensitivity and linearity testing methods used for the evaluation between handheld glucometers and RM were similar with the present study as such ROC (Mair et al. 2016), Bland-Altman plots and Passing-Bablok method comparison (Voyvoda et al. 2010; Mair et al. 2016; Mcmillan et al. 2017), except RMC analysis that was newly introduced in the present study. Furthermore, RMC showed the detailed performance of EDAN and GEM in hypoglycemia and hyperglycemia through DCP and ICP analysis, even in DSL.

Conclusion

The WBGC was systematically measured low by EDAN and high by GEM. The newly introduced RMC method confirmed this conclusion by showing detailed information about the test devices in high and low BGC at DCP and ICP. RMC can be used as an alternative method for the diagnostic accuracy of test devices. RMC showed that the diagnostic performance of GEM and EDAN was much lower, and both devices worked oppositely at low and high BGC. They were unable to differentiate the prevalence rate at critical DSL due to high misidentification of TP and TN samples. If conventional method comparison tests need to be applied in Holsteins, the biological fluctuation of BGC need to be taken into account because DSL changes the results of the methods. GEM and EDAN were not accurate for hypoglycemia and hyperglycemia diagnosis in dairy Holsteins. The logic and usefulness of the RMC method can be investigated using other devices that can accurately analyse BGC in dairy cows. As a next proposed study, repeated blood sampling in DSL in the same group of animals may help to clarify the variations in conventional statistical methods associated with RMC.

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