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Creatine kinase as marker for purulent vaginal discharge and fertility in beef cattle

Using creatine kinase to diagnose purulent vaginal discharge

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ABSTRACT

In beef cows, cycle is expected to resume within 30–35 days postpartum. Uterine diseases may delay these processes, extending the partum to conception to 50 days. Biomarkers for the diagnosis of Purulent vaginal discharge (PVD) in beef cows remain undefined. Creatine kinase (CK) has been investigated in dairy cows as a marker for clinical endometritis but not in beef cows. Mucus score and blood sampling were performed at 30 ± 5 in 264 non-pregnant Piedmontese beef cows and 28 of them were diagnosed with PVD. Cows that showed no successful conception after more than three AI with no apparent clinical disease were defined as repeat breeders ($N = 33$). Kruskal-Wallis test was used to detect difference in CK between cows with PVD and healthy ones and RB ($p = <.001$ and $p = .048$ respectively). No difference was found between healthy cows and RB ($p > .05$), cows PVD showed lower reproductive performances (PC and n°/IA) than healthy ones. Parity and farm didn't show differences between healthy and PVD cows. ROC curve was created to define a CK cut-off value for PVD detection (241 U/L, Sp 69%, Se 92%, AUC 0.81, Younden Index (J) 0.61) and to determine CK accuracy in predicting infertility at 120- and 150-days postpartum (Sp 77%, Se 42%, AUC 0.57, J 0.19 and Sp 82%, Se 34%, AUC 0.59, J 0.16 respectively). This study underlines the potential of CK as a marker of PVD in beef cows.

HIGHLIGHTS

- Creatine kinase seems to be a useful on field marker of PVD in beef cows
- Creatine kinase cut-off for PVD is 241 U/L
- Creatine kinase has low accuracy in prevision of infertility

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Piedmontese cow; purulent
vaginal discharge;
uterine pathology

Introduction

Beef cattle breeding is less standardised than dairy cattle because of the large number of different breeds and crossbreed and farming systems, ranging from intensive to extensive (Diskin and Kenny 2016). Moreover, the characteristics of some breeds are little investigated, thus low reproductive performances are often caused by failure of information about nutritional requirements, breeding, and farming management. Piedmontese beef cow is a high-quality double-muscling breed, due to a mutation of the myostatin gene (Kleiser and Fürll 1998) causing muscular hypertrophy. Piedmontese cows, as all double muscle breeds, are affected by a high incidence of dystocia and subsequent lower fertility and develop reproductive inflammatory conditions (Zaborski et al. 2009). In

general, a complete uterine involution requires about 30 days postpartum (dpp) and a total resumed oestrus cycle is expected to happen within 30–35 dpp in beef cows depending on breeds, nutritional status and on general managerial conditions (Diskin and Kenny 2016), but uterine conditions such as purulent vaginal discharge (PVD) can delay this process, causing economic damage to the farm (Williams et al. 2005; Bel and Roberts 2007; Dubuc et al. 2011). PVD is an inflammatory condition of the uterus associated with bacterial infection with purulent or muco-purulent uterine discharge with no systemic signs from 21 days after calving (Dubuc et al. 2010a, Ernstberger et al. 2019). However, not all the cows affected by uterine contamination postpartum will develop uterine disease (LeBlanc 2014), in fact the presence of PVD is a

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result of endometritis, cervicitis/vaginitis or the combination of both (Dubuc et al. 2011; Deguillaume et al. 2012) and the detrimental effects of endometritis and cervicitis/vaginitis on reproductive performances are additive (Sheldon et al. 2006). PVD incidence ranges from 15% to 60% in cows at 4–6 weeks postpartum due to differences in time of diagnosis, disease categorisation, and method used for the diagnosis and it has severe effects on fertility in both dairy and beef cows (LeBlanc et al. 2002; Ricci et al. 2017; Ryan et al. 2020). In general, cows affected by PVD need about 30 days more to become pregnant than unaffected cows (Dubuc et al. 2010b, 2011; Ricci et al. 2017). Early and non-invasive diagnosis of PVD is a key point to reduce partum to conception days (PC), in order to decrease the number of inseminations per pregnancy and improve reproductive performances (Dubuc et al. 2010a, 2010b). Assessment of PVD is normally performed through vaginoscopy, manual examination of the vagina, and Metrichick (LeBlanc 2008), whereas transrectal palpation of the uterus has lower predictive value for reproductive performances (Biswal et al. 2014; Ernstberger et al. 2019). Animals experiencing poor human-animal interaction, as it happens for Piedmontese cows, can show reactive behaviour and poor adaptation to handling and restraint, experiencing high levels of stress (Ceballos et al. 2018). The exam of the vaginal mucus requires the cleaning of the external genitalia and the manual collection of the mucus from the vaginal lumen. This process prolongs the clinical examination and could cause stress to the animals, making the restraint harder (LeBlanc 2014; Adnane et al. 2017). Therefore, blood sample could be an alternative diagnostic tool to evaluate uterine disease in cows.

Markers such as acute phase proteins (APPs) have been considered as indicators for general acute response, such as inflammation, tissue damage, and infection (Baumann and Gauldie 1994; Petersen et al. 2004) and stress (Hicks et al. 1998; Arthington et al. 2003; Hickey et al. 2003). Among APPs, haptoglobin has been suggested to serve as an indicator of PVD (Dubuc et al. 2010; Yasui et al. 2014), especially in the first two weeks postpartum (Pascottini and LeBlanc 2020). However, the use APPs as diagnostic biomarker is still debated for metritis and endometritis (Humblot et al. 2006; Azawi et al. 2008; Chang et al., 2010).

Creatine kinase (CK) serum concentrations have been investigated as a marker for PVD, showing different values in healthy and diseased cows (Sattler and Fürll 2004; McDougall et al. 2007). Creatine kinase is an intracellular cytosolic enzyme that catalyses the

reaction of creatine and adenosine triphosphate (ATP) to phosphocreatine and adenosine diphosphate (ADP) (Aujla and Patel 2020). It is a dimeric molecule composed of two subunits (M and B) and combinations of these subunits form the isoenzymes CK-MM, CK-MB, and CK-BB. CK is abundant in tissues with elevated energy transfer such as skeletal muscle, myocardium, and brain. In other visceral tissues (Cabaniss 1990), noticeable CK concentrations can be found in the uterine tissue and in every inner organ (Sattler and Fürll 2004). The serum of healthy cows contains almost entirely CK-MM, whereas inner organs contain mostly CK-BB. Mechanical and metabolic stress of the uterine tissue is known to cause elevated CK activities before and after normal parturition in cows (Abramov et al. 1996). Furthermore, serum concentrations of CK three days after parturition are lower in healthy Holstein cows (median of 121 U/L) than in cows with retained placenta (median 175 U/L), dystocia (median 310 U/L), milk fever (median of 385 U/L) (Kleiser and Fürll 1998), and abomasal displacement (Sattler and Fürll 2004). As for PVD, CK has been assessed in dairy cows (Sattler and Fürll 2004) and in Iraqi buffalo cows (Azawi et al. 2008) between 3 to 6 weeks pp. Results showed that animals with PVD had higher CK activity than healthy ones. To the best of our knowledge, CK has never been investigated as a diagnostic tool for PVD in beef cows.

The main objective of the present study was to evaluate the accuracy of CK serum concentrations in detecting PVD and in predicting infertility at 120 and 150-days postpartum. Moreover, we aimed to investigate the CK serum concentrations in repeat RB, that require three or more artificial inseminations (AI) without conception in the absence of clinical signs, representing a major reproductive issue in cows.

Materials and methods

The present study obtained the approval of the Ethical Committee of the Dipartimento di Scienze Veterinarie of the Università di Torino. All the included procedures did not interfere with the clinical management of the included animals and were performed in compliance with EU Directive 2010/63/CE. Treatment was always provided according to the clinical evaluation of the animals. Proper informed consent was obtained by the owners of the farms.

The present study was carried out in two farms of similar size (200 and 220 animals) with free stall barns and delivery parlour. All animals were fed with ad libitum feed (hay, bent grass, and corn flour and soya)

enriched with vitamins (A and E) and mineral supplementation (Ca, P and Mg). All animals were vaccinated for bovine viral diarrhoea (BVD) and infectious bovine rhinotracheitis (IBR). Both farms were officially free from tuberculosis, brucellosis and pneumonia.

A group of 264 non-pregnant Piedmontese cows was used to assess CK performances as a diagnostic tool for PVD. All cows underwent a first clinical examination at 30 ± 5 pp., including an investigation for PVD presence, in order to detect postpartum disease. Animals that presented other conditions such as lameness, pneumonia, and trauma were excluded from the study.

PVD was analysed by the gloved hand technique as described by Williams et al. (2005), using a 4-point classification system: 0 = no or clear mucus, 1 = mucus containing few flecks, 2 = discharge containing less than 50% pus, 3 = discharge containing more than 50% pus. Cows with a score of 0 or 1 were grouped as HEALTHY. Cows were considered diseased when scores were equal or higher than 2 (cut-off score = 2) (Williams et al. 2005) and they were grouped as 'PVD' and treated with one intrauterine infusion of 500 mg cephapirin benzathine (RCL) (Metricure, MSD Animal Health, Roma, ITALY) as proposed by Tison et al. 2017, and rechecked 7 days later. All cows underwent a pregnancy check at 30 ± 5 days after AI by ultrasound examination (Ibex[®] EVO II[®], E.I. Medical Imaging, Loveland, CO). Fertility data (PC and number of AI) were recorded retrospectively. Cows that showed more than three subsequent AI with regular cycles with no apparent clinical reproductive disease that did not show successful conception were defined as repeat breeder cows (RB).

Blood samples were collected at 30 ± 5 days pp. by venipuncture from the coccygeal vein using an 8 mL evacuated serum collection tube and a 20 G needle (Vacutainer[®] Venoject, Terumo, Leuven, Belgium). Blood samples were immediately refrigerated and transported to the laboratory within 4 hours. Blood was centrifuged at 2000 rpm for 10 min and the serum was separate and stored at -20°C in 1 mL SafeLock tubes (Eppendorf[®], Hamburg, Germany). Creatine kinase was measured with a clinical chemistry analyser KUADRO[®] BPC (Biosed s.r.l, Rimini, Italy) that uses Creatine Kinase immunologic kinetic UV-test (MTD Diagnostics, Caserta, Italy), in accordance with International Federation of Clinical Chemistry (IFCC) guidelines.

Statistical analysis

Individual animal data were manually collected from the computerised herd systems and recorded on

Microsoft Excel (Microsoft Corp., Redmond, WA) work file. Statistical analyses were performed using R statistical software (ver. 2.15.2, R Core Team, Vienna, Austria). p values $\leq .05$ were considered significant, and trends were considered at p values between .06 and .08.

Sample size calculation was performed (for two independent means) based on limited information available in the literature (Azawi et al. 2008, Sattler and Fürll 2004). R software was used (Package 'pwr') and means \pm DS, alpha of 0.05 and Power of 0.8 were used for the calculation.

Descriptive statistical analysis was performed to calculate the CK among all three experimental groups (HEALTHY, PVD, and RB). CK serum concentration was analysed using Kruskal-Wallis test considering the three animal groups (HEALTHY, RB, and PVD).

Furthermore, Kruskal-Wallis test was used to evaluate reproductive performances such as PC and number of AI per pregnancy among groups (HEALTHY, RB, and PVD). Bonferroni pot-hoc test was used for pairwise comparisons.

Receiver operating characteristic (ROC) curves (package pROC; Robin et al., 2011) was created and areas under the curve (AUC, package cvAUC) and Youden Index (J), calculated as $(\text{Se}(c) + \text{Sp}(c) - 1)$, were calculated to set the optimal serum CK concentration cut-off point to score PVD at 30 ± 5 days pp and to assess infertility in terms of PC at 120 and 150 days and number of AI.

Results and discussion

The aims of the present study were to investigate the serum CK concentrations as a marker for PVD during the postpartum and to assess differences in CK serum concentrations in RB cows.

From descriptive statistic 236 (89.4%, 236/264) cows were diagnosed as negative for PVD at the first clinical examination. However, 33 animals (13.9%, 33/236) were subsequently classified as RB when data were analysed retrospectively (RB group). Therefore, only 203 (76.9%, 203/264) animals were considered as healthy. Finally, twenty-eight (10.6%, 28/264) cows were diagnosed with PVD.

It is noticeable that, although Piedmontese cow is a double muscle breed and CK is abundant in muscular tissue, basal serum CK concentrations did not differ from the values reported in literature for dairy cows and Iraqi buffaloes (Sattler and Fürll 2004; Azawi et al. 2008). were higher in PVD positive Piedmontese beef cows than in Furthermore, in dairy cows the

Table 1. Serum Creatine kinase (CK) concentration, for healthy (HEALTHY), repeat breeder (RB), and diseased (PVD) cows.

	N.	CK (U/L)					
		Median	IQR ^a	p Value	Mean	SD	p Value
HEALTHY	203	196	122.2–268.5	$p < 0.001$	266	128	$p < 0.001$
RB	33	174	133.5–343		268	191	
PVD	28	346	282.2–635		448	263	

Healthy: not diseased cows; **RB (repeat breeder cows):** cows without clinical uterine disease with >3 AI after parturition. **PVD (purulent vaginal discharge):** cows positive for PVD using a 4-point classification system: 0=no or clear mucus, 1=mucus containing few flecks, 2=discharge containing less than 50% pus, 3=discharge containing more than 50% pus.

^aIQR: interquartile range.

possible interference of the postpartum period diseases and the influence of the oestrus, could be associated with higher mean CK serum concentrations (Sattler and Füll 2004; Crane et al. 2016). In this study, cows diagnosed with PVD showed an increase in serum CK concentrations when compared to HEALTHY ones ($p < .001$, Table 1). Furthermore, some of the animals that were negative to PVD were subsequently classified as repeat breeder cows (RB) because of the higher number of required AI. According to Salasel et al. (2010), incidence of RB ranges from 10% to 24% and many risk factors for repeat breeding have been described including parity, peri-parturient disease, uterine diseases, season, herd size, milk yield, and poor fertility (Perez-Marin et al. 2012). Furthermore, conditions such as subclinical endometritis (SCE), could increase the incidence of repeat breeder syndrome, being 52.7% of RB cows positive to SCE (Salasel et al. 2010; Ricci et al. 2015). In this study, intrauterine cytology has not been performed to investigate the presence of SCE in RB cows. Although, no data about CK values for SCE are available in the literature and because all RB cows in this study showed that serum CK concentration that did not differ from those of healthy cows, (Figure 1) then we can speculate that SCE does not influence the CK concentration in blood ($p > .05$). In this study differences for PC and AI were analysed among all groups (HEALTHY, RB, and PVD) with significant differences mainly in PVD cows (85 ± 35 , 191 ± 65 , 144 ± 27 , $p < .001$, Table 2). Parity and farms effects didn't show any difference ($p > .05$)

It is known that cows affected by PVD have a delay in conception compared to healthy ones (Ricci et al. 2015). Specifically, PVD has been indicated as detrimental on the reproductive performances of dairy cows and as previously mentioned, its incidence

ranges from 15% to 60% at 4–6 weeks postpartum in dairy cows depending on time of diagnosis, housing method, and it has severe effects on fertility in both dairy and beef cows (Deguillaume et al. 2012; Ricci et al. 2017; Ryan et al. 2020). It is commonly known that beef cows are not affected by remarkable metabolic imbalance and immunosuppression during the first and late postpartum, therefore, beef cows are expected to show a significantly lower incidence of PVD than dairy cows. In the present study 11% (28/264) of cows showed PVD, which is slightly lower than the one reported for dairy cows (10% to 35%) (Runciman et al. 2008; Deguillaume et al. 2012; de Boer et al. 2015). Nevertheless, no precise data about clinical uterine disease in beef cows is available in the literature.

PVD is commonly associated to current bacterial uterine infection, and it is a more practical routine cow-side method than cytological investigation (LeBlanc 2014).

Adnane et al. (2017) analysed cervico-vaginal mucus (CVM) as biomarker for clinical endometritis (CE), by performing cytology and assessing total protein and inflammatory biomarkers on CVM. Although, the collection through uterine washings by lavage expose to the risk of an unknown dilution factor. Nevertheless, Adnane et al. 2017 measured high levels of cytokines and other inflammatory biomarkers are successfully measured in CVM, suggesting that CVM may provide a more reliable sample for measuring inflammatory markers specific for the uterus. Both blood CK concentration measurement and CVM assessment require the collection of a sample on field and to have a laboratory to process them. Moreover, we think that using a simple blood marker with a specific cut-off is more feasible and easier on field than a more precise but more complex CVM assessment. Also, a blood sample require less material and the processing is cheaper than the analysis of CVM by cytology, total protein, and immunological pattern. Various acute phase proteins and blood metabolites have already been investigated in dairy and beef cows as inflammatory and stress response markers (Yasui et al. 2014; Pascottini and LeBlanc 2020). In accordance with other authors (Sattler and Füll 2004; Azawi et al. 2008), in our study serum CK concentrations increase more in cows with PVD than in healthy and repeat breeding cows.

As already mentioned, no data about serum CK concentrations in beef cows are available in the literature, therefore a ROC curve was created with the aim of

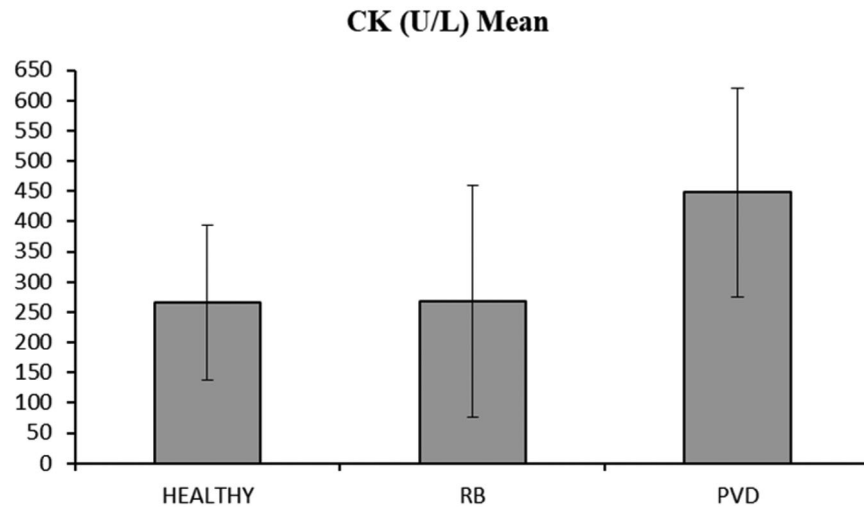


Figure 1. Creatine kinase (CK) concentration (mean \pm SD, U/L) in healthy, RB and PVD cows. **Healthy:** not diseased cows; **RB (repeat breeder cows):** cows without clinical uterine disease with >3 AI after parturition; **PVD (purulent vaginal discharge):** cows positive for PVD using a 4-point classification system: 0 = no or clear mucus, 1 = mucus containing few flecks, 2 = discharge containing less than 50% pus, 3 = discharge containing more than 50% pus.

Table 2. Partum-to-conception interval (PC), and number of artificial insemination per pregnancy (n AI/preg) for healthy (HEALTHY), repeat breeder (RB), and diseased (PVD) cows.

	N	PC gg			N AI/preg								
		Median	IQR	p Value	Mean	SD	p Value	Median	IQR ^a	p Value	Mean	SD	p Value
HEALTHY	203	77.5	60–104	$p < .001$	85	35	$p < .001$	2	01-mar	$p < .001$	1.8	0.84	$p < .001$
RB	33	179	132–238		191	65		5	04-giu		5.2	1.8	
PVD	28	142	130–160		144	27		3	2.75–4		3.1	0.8	

Healthy: not diseased cows; **RB (repeat breeder cows):** cows without clinical uterine disease with >3 AI after parturition; **PVD (purulent vaginal discharge):** cows positive for PVD using a 4-point classification system: 0 = no or clear mucus, 1 = mucus containing few flecks, 2 = discharge containing less than 50% pus, 3 = discharge containing more than 50% pus.

^aIQR: interquartile range.

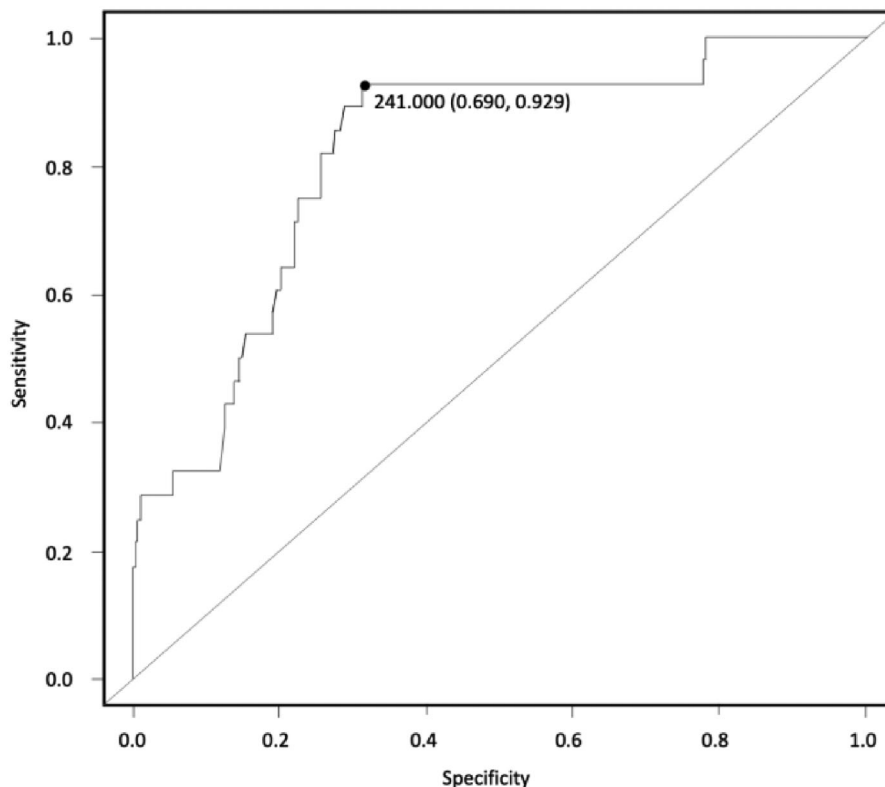


Figure 2. Creatine kinase (CK) blood concentration cut off for for PVD prediction. ROC curve (cut off indicates a cut-off of 241 U/L Se 92%, Sp 69%, AUC 0.81, J 61%).

Table 3. Receiver Operating Characteristics (ROC) curve results for serum Creatine kinase (CK) concentrations for detection of PVD and for prediction of infertility at 120- and 150-days postpartum.

	CK (U/L)	Sp%	Se%	AUC	IC	J %
PVD	241	69	92	0.81	0.73–0.89	0.61
PC120	286	77	42	0.57	0.49–0.55	0.19
PC150	341	82	34	0.59	0.47–0.65	0.16

PVD: Purulent vaginal discharge, PC120: Partum to conception at 120 days postpartum, PC150: partum to conception 150 days postpartum.

defining a cut-off value for the diagnosis of PVD in postpartum. As showed in Figure 2, the ROC curve for a precise diagnosis of PVD indicates a cut-off of 241 U/L for CK to predict PVD, showing good accuracy (Se 92%, Sp 69%, AUC 0.81, J 0.61%). The sensitivity of a test (true positive rate) is defined as the proportion of individuals with the disease who will have a positive result. Therefore, a highly sensitive test can be useful for ruling out a disease if an individual has a negative result (Petrie and Watson 2013). A highly specific test can be useful for ruling in patients who have a certain disease.

We have also found that serum CK at 30 ± 5 days postpartum is not a good predictor for infertility at 120 and 150-days postpartum (Table 3). The reason might be that infertility is not always strictly associated with inflammatory conditions or tissue damage (Weber et al. 2019; Moore and Biase 2020).

In conclusion, the results of this study underline the potential of CK as a cow-side marker for uterine disease in beef breeds, with the final goal to use serum CK as a good and fast method for the diagnosis of PVD mainly in conditions where manual on-field techniques are not easy to perform. Although at least 24 hours are needed for the results to be available due to the lack of portable readers, this could serve as a screening to determine which animals need more thorough investigations for PVD. Future studies should focus on investigating CK to assess its accuracy as a predictor of PVD at different postpartum times. Furthermore, the association between serum CK and SCE should be specifically investigated, performing cytology or CVM. Finally, a future goal could be the development of a quick tool for the assessment of blood CK on field, leading to a preventive and not invasive on-field diagnostic method, which could be implemented in the health check routine of postpartum cows.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All relevant data are within the manuscript and its Supporting Information files.

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