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

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Using creatine kinase to diagnose purulent vaginal discharge

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ABSTRACT

In beef cows, uterine involution requires about 30 days and oestrous cycles are expected to resume within 30-35 days postpartum depending on breeds. Uterine diseases may delay these processes and extend the partum to conception from 30 to 50 days. Uterine diseases have been associated with the repeat breeder syndrome in dairy cows. 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 in 264 non-pregnant Piedmontese beef cows from 25 to 35 days postpartum and 28 of them were diagnosed with PVD. Thirty-three cows were subsequently classified as repeat breeders (RB), all of them were negative to PVD. Kruskal-Wallis test was used to detect difference in CK between cows with PVD and heathy ones and RB ($P =<0.001$ and $P = 0.048$ respectively). No difference was found between healthy cows and RB ($P > 0.05$), 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.

Key words

Creatine kinase, Piedmontese cow, purulent vaginal discharge, uterine pathology

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

1. Introduction

Beef cattle breeding is less standardized 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-muscled 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). 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 result of endometritis, cervicitis/vaginitis or the combination of both (Deguillaume et al. 2012; Dubuc et al., 2011) 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 categorization, and method used for the diagnosis and it has severe effects on fertility in both dairy and beef cows (LeBlanc e 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; Dubuc et al., 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, Dubuc et al. 2010b). Assessment of PVD is normally performed through vaginoscopy, manual examination of the vagina, and Metricheck (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 restrain, 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 restrain harder (LeBlanc et al 2014). Therefore, blood sample could be an alternative diagnostic tool to evaluate uterine disease in cows.

Markers such as scute 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; Hickey et al., 2003; Arthington 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 et al., 2020). However, the use APPs as diagnostic biomarker is still debated for metritis and endometritis (Azawi et al., 2008, Hublet t al 2006, Cjang et al 2010).

Creatine kinase (CK) serum concentrations have been investigated as a marker for PVD, showing different values in healthy and diseased cows (McDougall et al., 2007; Sattler and Fürll, 2004). 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 (Cabannis, 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 (Pothmann et al. 2015), representing a major reproductive issue in cows.

2. 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 (100 and 120 animals) with free stall barns and delivery parlour. All animals were feed 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 from 25 to 35 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 were considered healthy (HEALTHY) with a score of 0 or 1. Cows were considered diseased when scores where 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, USA). 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) (Gustafsson and Emanuelson, 2002).

Blood samples were collected by venipuncture from the coccygeal vein using an 8 mL evacuated serum collection tube and a 20 G needle (Vacutainer® Venoject, Terumo, Leueven, Belgium). Blood samples were immediately refrigerated and transported to the laboratory within 4 hours. Blood was centrifuged at 2,000 rpm for 10 minutes 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.

Individual animal data were manually collected from the computerized 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 ≤ 0.05 were considered significant, and trends were considered at P values between 0.06 and 0.08.

Sample size calculation was performed (for two independent means) based on limited information available in the literature (Azawi et al 2008, Settler and furl 2004) . R software was used (Package "pwr") and means ± 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 partum-to-conception interval (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 (Se(c) + 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.

3. 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 Iraqui buffaloes (Sattler and Fürll, 2004;Azawi et al. 2008). were higher in PVD positive Piedmontese beef cows than in Furthermore, in dairy cows the possible interference of the postpartum period diseases and the influence of the oestrus, could be associated with higher mean CK serum concentrations (Sattler and Fürll, 2004; Crane et al., 2016). In this study, cows diagnosed with PVD showed an increase in serum CK concentrations when compared to HEALTHY ones ($P<0.001$, Table 1). Furthermore, some of

188 the animals that were negative to PVD were subsequently classified as repeat breeder cows
189 (RB) because of the higher number of required AI. According to Salasel et al. (2010),
190 incidence of RB ranges from 10 to 24% and many risk factors for repeat breeding have been
191 described including parity, peri-parturient disease, uterine diseases, season, herd size, milk
192 yield, and poor fertility (Perez-Marin et al. 2012). Furthermore, conditions such as subclinical
193 endometritis (SCE), could increase the incidence of repeat breeder syndrome, being 52.7% of
194 RB cows positive to SCE (Salasel et al. 2010; Ricci et al., 2015). In this study, intrauterine
195 cytology has not been performed to investigate the presence of SCE in RB cows. Although,
196 no data about CK values for SCE are available in the literature and because all RB cows in
197 this study showed that serum CK concentration that did not differ from those of healthy cows,
198 (Graph 1) then we can speculate that SCE does not influence the CK concentration in blood
199 ($P>0.05$). In this study differences for PC and AI were analysed among all groups
200 (HEALTHY, RB, and PVD) with significant differences mainly in PVD cows (Table 2).
201 Parity and farms effects didn't' show any difference ($P>0.05$)
202 It is known that cows affected by PVD have a delay in conception compared to healthy ones
203 (Ricci et al., 2015). Specifically, PVD has been indicated as detrimental on the reproductive
204 performances of dairy cows and as previously mentioned, its incidence ranges from 15% to
205 60% at 4-6 weeks postpartum in dairy cows depending on time of diagnosis, housing method,
206 and it has severe effects on fertility in both dairy and beef cows (Deguillaume et al., 2012;
207 Ricci et al., 2017, Rayan et al 2020). It is commonly known that beef cows are not affected by
208 remarkable metabolic imbalance and immunosuppression during the first and late postpartum,
209 therefore, beef cows are expected to show a significantly lower incidence of PVD than dairy
210 cows. In the present study 11% (28/264) of cows showed PVD, which is slightly lower than
211 the one reported for dairy cows (10% to 35%) (Ruciman et al 2008, Deguillaume et al., 2012,

212 deBoer et al 2015). Nevertheless, no precise data about clinical uterine disease in beef cows is
213 available in the literature.
214 PVD is commonly associated to current bacterial uterine infection, and it is a more practical
215 routine cow-side method than cytological investigation (LeBlanc et al 2014).
216 Adnane et al. (2017) analysed cervico-vaginal mucus (CVM) as biomarker for clinical
217 endometritis (CE), by performing cytology and assessing total protein and inflammatory
218 biomarkers on CVM. Although, the collection trough uterine washings by lavage requires
219 perfusion of solution into the uterus, with the risk of an unknown dilution factor and with the
220 difficulty to recover the total volume of infused solution. Neverless , Adnane et al 2017
221 measured high levels of cytokines and other inflammatory biomarkers are successfully
222 measured in CVM, suggesting that CVM may provide a more reliable sample for measuring
223 inflammatory markers specific for the uterus. Both blood CK concentration measurement and
224 CVM assessment require the collection of a sample on field and to have a laboratory to
225 process them. Moreover we think that using a simple blood marker with a specific cut-off is
226 more feasible and easier on field than a more precise but more complex CVM assessment.
227 Also, a blood sample require less material and the processing is cheaper than the analysis of
228 CVM by cytology, total protein, and immunological pattern. Various acute phase proteins and
229 blood metabolites have already been investigated in dairy and beef cows as inflammatory and
230 stress response markers (Pascottini et al., 2020; Yasui et al., 2014). In accordance with other
231 authors (Azawi et al., 2007; Sattler and Fürll, 2004), in our study serum CK concentrations
232 increase more in cows with PVD than in healthy and repeat breeding cows.
233 As already mentioned, no data about serum CK concentrations in beef cows are available in
234 the literature, therefore a ROC curve was created with the aim of defining a cut-off value for
235 the diagnosis of PVD in postpartum. As showed in Figure 1, the ROC curve for a precise
236 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 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 (Moorey and Biase, 2020; Weber et al., 2019).

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 were manual on-field techniques are not easy to perform. 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.

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DECLARATION OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

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

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

		CK (U/L)					
	N.	**Median**	**IQR**	**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.

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.

		PC gg						N AI/preg					
	N.	**Median**	**IQR**	**P value**	**Mean**	**SD**	**P value**	**Median**	**IQR**	**P value**	**Mean**	**SD**	**P value**
HEALTHY	203	77.5	60-104	P<0.001	85	35	P<0.001	2	01-mar	P<0.001	1.8	0.84	P<0.001
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.

429 **Graph 1** CK concentration (mean ± SD, U/L) in healthy, RB and PVD cows

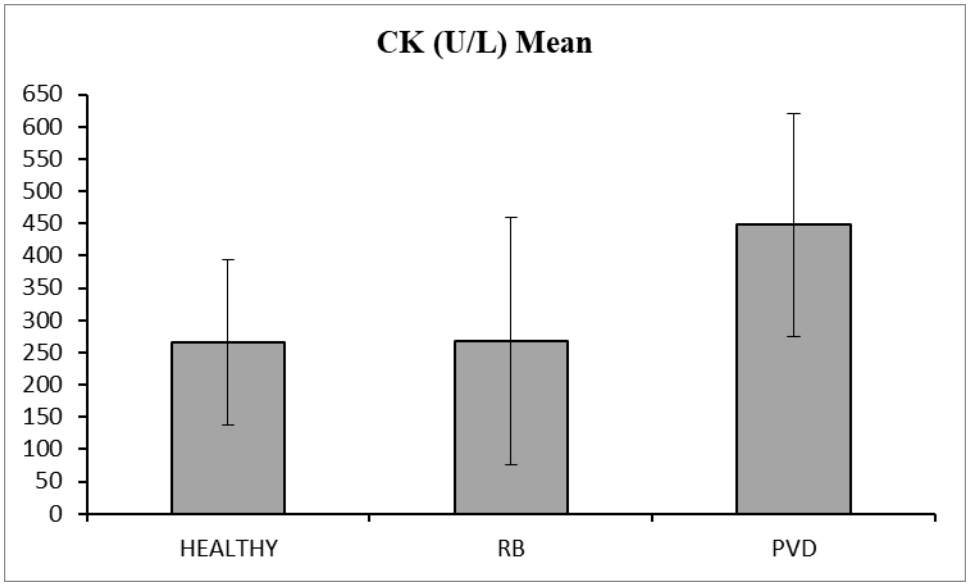

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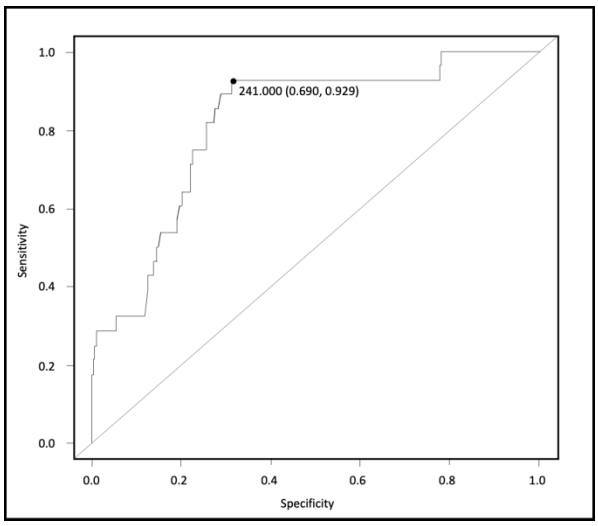
Table 3 – Receiver Operating Characteristics (ROC) curve results for serum CK concentrations for detection of PVD and for prediction of infertility at 120- and 150-days postpartum.

	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.



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Graph 2 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%)