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

2 Using creatine kinase to diagnose purulent vaginal discharge

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14 **ABSTRACT**

15 In beef cows, uterine involution requires about 30 days and oestrous cycles are expected to
16 resume within 30-35 days postpartum depending on breeds. Uterine diseases may delay these
17 processes and extend the partum to conception from 30 to 50 days. Uterine diseases have been
18 associated with the repeat breeder syndrome in dairy cows. Biomarkers for the diagnosis of
19 Purulent vaginal discharge (PVD) in beef cows remain undefined. Creatine kinase (CK) has
20 been investigated in dairy cows as a marker for clinical endometritis but not in beef cows.
21 Mucus score and blood sampling were performed in 264 non-pregnant Piedmontese beef
22 cows from 25 to 35 days postpartum and 28 of them were diagnosed with PVD. Thirty-three
23 cows were subsequently classified as repeat breeders (RB), all of them were negative to PVD.
24 Kruskal-Wallis test was used to detect difference in CK between cows with PVD and healthy
25 ones and RB ($P = <0.001$ and $P = 0.048$ respectively). No difference was found between
26 healthy cows and RB ($P > 0.05$), cows PVD showed lower reproductive performances (PC
27 and n°/IA) than healthy ones. Parity and farm didn't show differences between healthy and
28 PVD cows. ROC curve was created to define a CK cut-off value for PVD detection (241 U/L,
29 Sp 69%, Se 92%, AUC 0.81, Younden Index (J) 0.61) and to determine CK accuracy in
30 predicting infertility at 120- and 150-days postpartum (Sp 77%, Se 42%, AUC 0.57, J 0.19
31 and Sp 82%, Se 34%, AUC 0.59, J 0.16 respectively). This study underlines the potential of
32 CK as a marker of PVD in beef cows.

33

34 **Key words**

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

36 **Highlights**

- 37
- 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
- 38

39 • Creatine kinase has low accuracy in prevision of infertility

40

41 **1. Introduction**

42 Beef cattle breeding is less standardized than dairy cattle because of the large number of
43 different breeds and crossbreed and farming systems, ranging from intensive to extensive
44 (Diskin and Kenny, 2016). Moreover, the characteristics of some breeds are little investigated,
45 thus low reproductive performances are often caused by failure of information about
46 nutritional requirements, breeding, and farming management. Piedmontese beef cow is a
47 high-quality double-muscléd breed, due to a mutation of the myostatin gene (Kleiser and
48 Füll, 1998) causing muscular hypertrophy. Piedmontese cows, as all double muscle breeds,
49 are affected by a high incidence of dystocia and subsequent lower fertility and develop
50 reproductive inflammatory conditions (Zaborski et al. 2009). In general, a complete uterine
51 involution requires about 30 days postpartum (dpp) and a total resumed oestrus cycle is
52 expected to happen within 30-35 dpp in beef cows depending on breeds, nutritional status and
53 on general managerial conditions (Diskin and Kenny, 2016), but uterine conditions such as
54 purulent vaginal discharge (PVD) can delay this process, causing economic damage to the
55 farm (Williams et al 2005). PVD is an inflammatory condition of the uterus associated with
56 bacterial infection with purulent or muco-purulent uterine discharge with no systemic signs
57 from 21 days after calving (Dubuc et al 2010a, Ernstberger et al., 2019). However, not all the
58 cows affected by uterine contamination postpartum will develop uterine disease (LeBlanc
59 2014), in fact the presence of PVD is a result of endometritis, cervicitis/vaginitis or the
60 combination of both (Deguillaume et al. 2012; Dubuc et al., 2011) and the detrimental effects
61 of endometritis and cervicitis/vaginitis on reproductive performances are additive (Sheldon et
62 al., 2006). PVD incidence ranges from 15 to 60% in cows at 4-6 weeks postpartum due to
63 differences in time of diagnosis, disease categorization, and method used for the diagnosis
64 and it has severe effects on fertility in both dairy and beef cows (LeBlanc et al., 2002; Ricci et
65 al., 2017, Ryan et al 2020). In general, cows affected by PVD need about 30 days more to

66 become pregnant than unaffected cows (Dubuc et al., 2010b; Dubuc et al., 2011; Ricci et al.,
67 2017). Early and non-invasive diagnosis of PVD is a key point to reduce partum to
68 conception days (PC), in order to decrease the number of inseminations per pregnancy and
69 improve reproductive performances (Dubuc et al. 2010a, Dubuc et al. 2010b). Assessment of
70 PVD is normally performed through vaginoscopy, manual examination of the vagina, and
71 Metricheck (LeBlanc, 2008), whereas transrectal palpation of the uterus has lower predictive
72 value for reproductive performances (Biswal et al., 2014; Ernstberger et al., 2019). Animals
73 experiencing poor human-animal interaction, as it happens for Piedmontese cows, can show
74 reactive behaviour and poor adaptation to handling and restrain, experiencing high levels of
75 stress (Ceballos et al., 2018). The exam of the vaginal mucus requires the cleaning of the
76 external genitalia and the manual collection of the mucus from the vaginal lumen. This
77 process prolongs the clinical examination and could cause stress to the animals, making the
78 restrain harder (LeBlanc et al 2014). Therefore, blood sample could be an alternative
79 diagnostic tool to evaluate uterine disease in cows.

80 Markers such as acute phase proteins (APPs) have been considered as indicators for general
81 acute response, such as inflammation, tissue damage, and infection (Baumann and Gauldie,
82 1994; Petersen et al., 2004) and stress (Hicks et al., 1998; Hickey et al., 2003; Arthington et
83 al., 2003). Among APPs, haptoglobin has been suggested to serve as an indicator of PVD
84 (Dubuc et al., 2010; Yasui et al., 2014), especially in the first two weeks postpartum
85 (Pascottini et al., 2020). However, the use APPs as diagnostic biomarker is still debated for
86 metritis and endometritis (Azawi et al., 2008, Hublet et al 2006, Cjang et al 2010).

87 Creatine kinase (CK) serum concentrations have been investigated as a marker for PVD,
88 showing different values in healthy and diseased cows (McDougall et al., 2007; Sattler and
89 Füll, 2004). Creatine kinase is an intracellular cytosolic enzyme that catalyses the reaction of
90 creatine and adenosine triphosphate (ATP) to phosphocreatine and adenosine diphosphate

91 (ADP) (Aujla and Patel, 2020). It is a dimeric molecule composed of two subunits (M and B)
92 and combinations of these subunits form the isoenzymes CK-MM, CK-MB, and CK-BB.
93 CK is abundant in tissues with elevated energy transfer such as skeletal muscle, myocardium,
94 and brain. In other visceral tissues (Cabannis, 1990), noticeable CK concentrations can be
95 found in the uterine tissue and in every inner organ (Sattler and Fürll, 2004). The serum of
96 healthy cows contains almost entirely CK-MM, whereas inner organs contain mostly CK-BB.
97 Mechanical and metabolic stress of the uterine tissue is known to cause elevated CK activities
98 before and after normal parturition in cows (Abramov et al., 1996). Furthermore, serum
99 concentrations of CK three days after parturition are lower in healthy Holstein cows (median
100 of 121 U/l) than in cows with retained placenta (median 175 U/l), dystocia (median 310 U/l),
101 milk fever (median of 385 U/l) (Kleiser and Fürll, 1998), and abomasal displacement (Sattler
102 and Fürll, 2004). As for PVD, CK has been assessed in dairy cows (Sattler and Fürll, 2004)
103 and in Iraqi buffalo cows (Azawi et al., 2008) between 3 to 6 weeks pp. Results showed that
104 animals with PVD had higher CK activity than healthy ones. To the best of our knowledge,
105 CK has never been investigated as a diagnostic tool for PVD in beef cows.
106 The main objective of the present study was to evaluate the accuracy of CK serum
107 concentrations in detecting PVD and in predicting infertility at 120 and 150-days postpartum.
108 Moreover, we aimed to investigate the CK serum concentrations in repeat RB, that require
109 three or more artificial inseminations (AI) without conception in the absence of clinical signs
110 (Pothmann et al. 2015), representing a major reproductive issue in cows.

111

112 **2. Materials and methods**

113 The present study obtained the approval of the Ethical Committee of the Dipartimento di
114 Scienze Veterinarie of the Università di Torino. All the included procedures did not interfere
115 with the clinical management of the included animals and were performed in compliance with

116 EU Directive 2010/63/CE. Treatment was always provided according to the clinical
117 evaluation of the animals. Proper informed consent was obtained by the owners of the farms.
118 The present study was carried out in two farms of similar size (100 and 120 animals) with free
119 stall barns and delivery parlour. All animals were feed with ad libitum feed (hay, bent grass,
120 and corn flour and soya) enriched with vitamins (A and E) and mineral supplementation (Ca,
121 P and Mg). All animals were vaccinated for bovine viral diarrhoea (BVD) and infectious
122 bovine rhinotracheitis (IBR). Both farms were officially free from tuberculosis, brucellosis
123 and pneumonia.

124 A group of 264 non-pregnant Piedmontese cows was used to assess CK performances as a
125 diagnostic tool for PVD. All cows underwent a first clinical examination from 25 to 35 pp,
126 including an investigation for PVD presence, in order to detect postpartum disease. Animals
127 that presented other conditions such as lameness, pneumonia, and trauma were excluded from
128 the study.

129 PVD was analysed by the gloved hand technique as described by Williams et al. (2005), using
130 a 4-point classification system: 0 = no or clear mucus, 1 = mucus containing few flecks,
131 2 = discharge containing less than 50% pus, 3 = discharge containing more than 50% pus.

132 Cows were considered healthy (HEALTHY) with a score of 0 or 1. Cows were considered
133 diseased when scores where equal or higher than 2 (cut-off score = 2) (Williams et al., 2005)
134 and they were grouped as 'PVD' and treated with one intrauterine infusion of 500 mg
135 cephapirin benzathine (RCL) (Metricure, MSD Animal Health, Roma, ITALY) as proposed
136 by Tison et al. 2017, and rechecked 7 days later. All cows underwent a pregnancy check at
137 30±5 days after AI by ultrasound examination (Ibex® EVO II®, E.I. Medical Imaging,
138 Loveland CO, USA). Fertility data (PC and number of AI) were recorded retrospectively.
139 Cows that showed more than three subsequent AI with regular cycles with no apparent

140 clinical reproductive disease that did not show successful conception were defined as repeat
141 breeder cows (RB) (Gustafsson and Emanuelson, 2002).

142 Blood samples were collected by venipuncture from the coccygeal vein using an 8 mL
143 evacuated serum collection tube and a 20 G needle (Vacutainer[®] Venoject, Terumo, Leuven,
144 Belgium). Blood samples were immediately refrigerated and transported to the laboratory
145 within 4 hours. Blood was centrifuged at 2,000 rpm for 10 minutes and the serum was
146 separate and stored at -20°C in 1 mL SafeLock tubes (Eppendorf[®], Hamburg, Germany).

147 Creatine kinase was measured with a clinical chemistry analyser KUADRO[®] BPC (Biosed
148 s.r.l, Rimini, Italy) that uses Creatine Kinase immunologic kinetic UV-test (MTD
149 Diagnostics, Caserta, Italy), in accordance with International Federation of Clinical Chemistry
150 (IFCC) guidelines.

151 Individual animal data were manually collected from the computerized herd systems and
152 recorded on Microsoft Excel (Microsoft Corp., Redmond, WA) work file. Statistical analyses
153 were performed using R statistical software (ver. 2.15.2, R Core Team, Vienna, Austria). P
154 values ≤ 0.05 were considered significant, and trends were considered at P values between
155 0.06 and 0.08.

156 Sample size calculation was performed (for two independent means) based on limited
157 information available in the literature (Azawi et al 2008, Settler and furl 2004) . R software
158 was used (Package “pwr”) and means \pm DS, alpha of 0.05 and Power of 0,8 were used for the
159 calculation.

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

163 Furthermore, Kruskal-Wallis test was used to evaluate reproductive performances such as
164 partum-to-conception interval (PC) and number of AI per pregnancy among groups
165 (HEALTHY, RB, and PVD). Bonferroni pot-hoc test was used for pairwise comparisons.
166 Receiver operating characteristic (ROC) curves (package pROC; Robin et al., 2011) was
167 created and areas under the curve (AUC, package cvAUC) and Youden Index (J), calculated
168 as $(Se(c) + Sp(c) - 1)$, were calculated to set the optimal serum CK concentration cut-off point
169 to score PVD at 30 ± 5 days pp and to assess infertility in terms of PC at 120 and 150 days and
170 number of AI.

171 **3. Results and discussion**

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

175 From descriptive statistic 236 (89.4%, 236/264) cows were diagnosed as negative for PVD at
176 the first clinical examination. However, 33 animals (13.9%, 33/236) were subsequently
177 classified as RB when data were analysed retrospectively (RB group). Therefore, only 203
178 (76.9%, 203/264) animals were considered as healthy. Finally, twenty-eight (10.6%, 28/264)
179 cows were diagnosed with PVD.

180 It is noticeable that, although Piedmontese cow is a double muscle breed and CK is abundant
181 in muscular tissue, basal serum CK concentrations did not differ from the values reported in
182 literature for dairy cows and Iraqui buffaloes (Sattler and Fürll, 2004; Azawi et al. 2008).
183 were higher in PVD positive Piedmontese beef cows than in Furthermore, in dairy cows the
184 possible interference of the postpartum period diseases and the influence of the oestrus, could
185 be associated with higher mean CK serum concentrations (Sattler and Fürll, 2004; Crane et
186 al., 2016). In this study, cows diagnosed with PVD showed an increase in serum CK
187 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 through 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. Nevertheless, 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

237 accuracy (Se 92%, Sp 69%, AUC 0.81, J 61%). The sensitivity of a test (true positive rate) is
238 defined as the proportion of individuals with the disease who will have a positive result.
239 Therefore, a highly sensitive test can be useful for ruling out a disease if an individual has a
240 negative result (Petrie and Watson, 2013). A highly specific test can be useful for ruling in
241 patients who have a certain disease.

242 We have also found that serum CK at 30±5 days postpartum is not a good predictor for
243 infertility at 120 and 150-days postpartum (Table 3). The reason might be that infertility is not
244 always strictly associated with inflammatory conditions or tissue damage (Moorey and Biase,
245 2020; Weber et al., 2019).

246 In conclusion, the results of this study underline the potential of CK as a cow-side marker for
247 uterine disease in beef breeds, with the final goal to use serum CK as a good and fast method
248 for the diagnosis of PVD mainly in conditions where manual on-field techniques are not easy
249 to perform. Future studies should focus on investigating CK to assess its accuracy as a
250 predictor of PVD at different postpartum times. Furthermore, the association between serum
251 CK and SCE should be specifically investigated, performing cytology or CVM . Finally, a
252 future goal could be the development of a quick tool for the assessment of blood CK on field,
253 leading to a preventive and not invasive on-field diagnostic method, which could be
254 implemented in the health check routine of postpartum cows.

255

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259

260 **DECLARATION OF INTEREST STATEMENT**

261 The authors declare no conflict of interest.

262 **DATA AVAILABILITY STATEMENT**

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

264

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

	N.	CK (U/L)					
		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	

419

420 **Healthy:** not diseased cows; **RB (repeat breeder cows):** cows without clinical uterine disease with >3 AI after parturition **PVD (purulent vaginal discharge):** cows

421 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

422 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	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	

423

424 **Healthy:** not diseased cows; **RB (repeat breeder cows):** cows without clinical uterine disease with >3 AI after parturition **PVD (purulent vaginal discharge):** cows

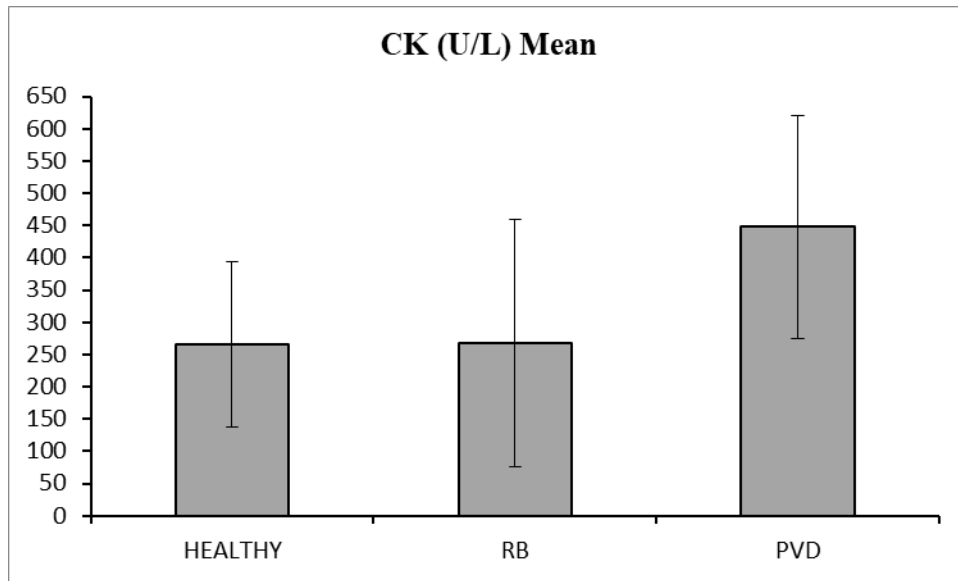
425 positive for PVD using a 4-point classification system: 0 = no or clear mucus, 1 = mucus containing few flecks, 2 = discharge containing less than 50% pus, 3 = discharge

426 containing more than 50% pus.

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

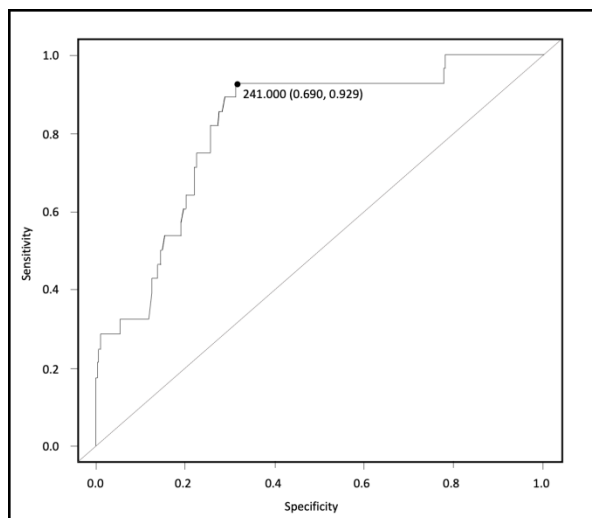


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Table 3 – Receiver Operating Characteristics (ROC) curve results for serum CK concentrations for detection of PVD and for prediction of infertility at 120- and 150-days postpartum.

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

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