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Investigating the Accuracy of Rapid P4 Milk Progesterone Measurement Kit for Detecting Estrus and Pregnancy in Dairy Cows and Comparing it with Other Conventional Methods

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Abstract: *Accuracy and precision in diagnosing estrus, time of insemination and early detection of pregnancy are among the main factors for a successful dairy cattle reproduction system. This study was implemented to evaluate the accuracy of Rapid P4 milk progesterone measurement kit for detecting estrus and pregnancy in dairy cows and comparing them with other conventional methods in the northeast of Iran. The study was performed on 30 Holstein cattle in the second and third calvings in Golestan province (Gonbad) and North Khorasan Province (Bojnourd) to investigate the accuracy and precision of P4 kit for detecting estrus and pregnancy in cows. During the study, the cattle were examined and verified for estrus and artificial insemination was carried out on them. On days 19, 20, 22, and 24 following the artificial insemination, milk samples were taken from the cattle to perform P4 tests, as well as blood samples to measure estrogen and progesterone in blood and milk using Dia metra commercial kit (Italy, Progesterone Elisa, and Estradiol Elisa). Out of 30 inseminated cattle, 7 had a progesterone level of less than 2 ng/ml in plasma and milk, which were marked as low progesterone samples by Rapid P4 test, and all of them showed estrus eventually. Although ultrasound and rectal examination verified pregnancy in days 35 and 45 following the insemination in 23 cattle, 3 were marked as non-pregnant by P4 test and 20 were diagnosed as true pregnant cows. In this case, the accuracy and precision of P4 kit in detecting true pregnancy was 87% and regarding the false pregnancy, it was 13%. Therefore, determining the amount of steroid hormones using ELISA or commercial kits can be used for assessing the reproductive status of dairy cows.*

Keywords: Progesterone, Rapid P4, Corpus luteum, Estrus, Estrogen

INTRODUCTION

Livestock sources are considered as national capitals for every country, and dairy industry is among the most considered resources due to reproductive efficiency and diverse products, as well as providing protein needs of the societies. One of the most important aspects of reproductive management is timely detection of estrus and pregnancy in cows, which is of utmost importance (Parham and Tavassolian, 2014; Dirandeh, 2008). The rate of pregnancy is measured using factors of

detecting estrus and true pregnancies (Parham and Tavassolian, 2014; Zamiri, 2002). Since the method of checking cows, 3 times a day for detecting estrus, is time-consuming, diagnosis of pregnancy and estrus by measuring progesterone and estrogen in milk and blood are introduced for early detection of pregnancy and estrus in cattle. In this study, the accuracy of P4 kits (made by England) in the detection of early pregnancy and estrus was assessed in cows and was compared with other common methods.

Periods of estrus cycle:

The estrus cycle involves four periods: proestrus, estrus, metestrus, and diestrus. The first and second periods are the follicular phases of the ovarian cycle and the third and fourth periods are the luteal phases (Zamiri, 2006). Detection of estrus using standing estrus (heat) sign is important especially when the artificial insemination program is programmed to be done. The inability to determine estrus cattle for performing insemination in optimal time range is a major dilemma for the dairy industry in many countries (Parham and Tavassolian, 2014; Mojabi, 2000).

Estrus behaviors in cattle and detecting estrus cows:

Three different patterns are observed during the estrus period including A. Cow's desire to perform mounting on other cows, B. Standing of the female animal moveless and waiting for the male cow to mount on it, which indicates a definite estrus. Standing up of the cow in the position of being mounted is the most desirable way to confirm heat (Mahmoudzadeh, 2001). C. Pug sign on the back and the secretions from the vagina which are also known as definite signs of heat (Mahmoudzadeh, 2001; Hashemi and Hasani, 2001).

Structure of progesterone hormone:

Progesterone is a sex hormone in the cattle which is a 21-carbon steroid. Progesterone is secreted from the corpus luteum during the normal ovulation cycle, and the discontinuation in its secretion leads to the symptoms of estrus cattle. A large amount of this hormone is secreted during pregnancy through placenta, which leads to the continuation of the pregnancy. A small amount of hormone is secreted from the adrenal glands as well. This hormone can be measured in the milk and blood of the livestock for detecting pregnancy and estrus (Zamiri, 2002; Zamiri, 2006).

Detecting progesterone (P4) in milk:

The level of P4 is higher in milk compared to plasma and it is a practical solution for a large number of cows, which could be considered as a potential method for future in detecting estrus if done manually. Using a developed automatic biometric sensor kit program, which includes Elisa, is like a transmission mechanism. Although it has a high price, it can control progesterone during each lactation and can be very valuable for identifying progesterone and the detection of estrus in cows (Mahmoudzadeh, 2001).

Detecting estrus using Estradiol-B17 in milk:

The Enzyme Immunoassay Test (EIA) is reliable for determining pre-ovulation Estradiol in raw milk samples, as well as cattle with P4 isolate. This method is reliable, rapid, cost-effective and accurate for determining the cow's estrus (Mahmoudzadeh, 2001; Domenech et al., 2011).

Measuring progesterone concentration:

Measurement of progesterone concentration can be done to detect pregnancy on day 21 after artificial insemination (and even earlier). Between days 21 to 24 after ovulation, the concentration of progesterone becomes high in blood and milk of pregnant cows, while it becomes absolutely low in non-pregnant cows and at the base level. Therefore, measuring the concentration of progesterone in milk samples obtained within this time range can help to distinguish non-pregnant cows (Zamiri, 2006; Donaldson et al., 1970).

Materials and methods

Research location:

The study was conducted on 30 Holstein cattles with two and three calvings in Golestan (Gonbad) and North Khorasan Provinces (Bojnourd) in order to assess the accuracy and precision of P4 kit in detecting estrus and pregnancy in cows.

Preparation of samples and methodology:

After identification, examination and assurance of the health of the genital system, the selected cows for the study were marked by ear tags, and they were inseminated artificially after detection of estrus. On days 19, 20, 22, and 24 following the artificial insemination, samples were taken from the milk for conducting P4 test, and measuring of estrogen and progesterone was done in blood and milk samples using Dia metra (Italy, Progesterone Elisa, and Estradiol Elisa). The criteria for approving pregnancy in this study was the progesterone concentration in milk and plasma, which, according to studies, concentrations higher than 2 ng/ml could be marked as pregnant and concentrations less than 2 ng/ml were considered non-pregnant. Results from measuring progesterone in milk and blood plasma have been presented in Table (1).

Table 1: Measurement of progesterone in milk and blood plasma in cows using ELISA method on days 0, 19, 20, 22, and 24 following the artificial insemination in nanograms per milliliters

Ear tag Number	Calvings	Estrus cycle 0 days		Estrus cycle 19 days		Estrus cycle 20 days		Estrus cycle 22 days		Estrus cycle 24 days	
1	2	Milk	Plasma	Milk	Plasma	Milk	Plasma	Milk	Plasma	Milk	Plasma
		1.68	1.42	4.32	3.90	4.47	3.98	4.62	4.32	4.95	4.67
2	2	1.22	1.01	3.78	3.27	4.17	3.82	4.45	4.13	4.90	4.30
3	2	1.92	1.78	3.61	3.07	4.18	3.92	4.72	3.99	5.01	4.07
*4	2	1.68	1.23	2.51	2.17	1.63	1.43	2.41	2.16	2.82	2.35
5	2	1.73	1.42	4.08	3.72	4.42	3.95	4.95	4.70	5.17	5.21
6	3	1.89	1.88	4.27	3.98	4.68	4.11	4.93	4.18	5.23	4.33
7	3	1.92	1.67	3.92	3.17	4.41	4.20	4.73	4.32	4.95	4.37
8	3	1.84	1.55	4.98	4.70	5.12	4.75	5.34	4.92	5.41	5.01
*9	3	1.68	1.43	2.79	2.47	1.92	1.52	2.77	2.48	2.89	2.63
10	3	1.90	1.72	3.93	3.64	4.22	3.97	4.48	4.02	4.63	4.11
11	2	1.72	1.35	4.07	3.75	4.34	3.88	4.51	3.92	4.92	4.17
12	2	1.85	1.42	4.83	4.11	5.12	4.27	5.17	4.30	5.35	4.36
13	2	1.67	1.12	3.12	2.98	3.02	2.74	3.38	3.02	3.42	3.14
*14	2	1.72	1.28	2.17	1.90	1.97	1.72	2.58	2.34	2.76	2.61
15	2	1.85	1.14	3.82	3.41	4.17	3.62	4.25	3.87	4.29	3.92
16	3	1.59	1.19	4.52	4.09	4.83	4.28	5.07	4.33	5.21	4.37
17	3	1.75	1.37	4.99	4.70	5.13	4.78	5.62	4.83	5.94	4.91
18	3	1.94	1.63	3.37	3.07	3.28	2.92	3.19	2.88	3.24	2.97
19	3	1.85	1.22	4.77	4.29	4.93	4.32	5.14	4.41	5.65	4.71
*20	3	1.62	1.39	2.85	2.37	1.65	1.65	2.70	2.22	2.78	2.25

*21	**2**	1.76	1.27	2.82	2.48	1.77	1.53	2.69	2.51	2.74	2.63
22	**2**	1.83	1.14	3.12	2.90	3.62	3.18	3.29	3.02	3.30	3.18
23	**2**	1.95	1.28	4.32	4.12	4.77	4.27	5.11	4.32	5.62	4.44
*24	**2**	1.88	1.31	2.63	2.41	1.40	1.23	2.37	2.12	2.52	2.28
25	**2**	1.62	1.43	4.61	4.08	4.88	4.32	5.09	4.38	5.21	4.49
26	**2**	1.52	1.30	4.28	3.86	4.77	3.95	5.19	4.11	5.21	4.18
*27	**2**	1.48	1.22	2.78	2.38	1.52	1.23	2.89	2.54	2.93	2.62
28	**2**	1.82	1.18	4.88	4.02	4.89	4.77	5.83	4.92	5.92	5.03
29	**2**	1.67	1.21	4.15	3.72	4.41	3.96	4.88	4.11	5.02	4.62
30	**2**	1.42	1.37	4.81	4.18	4.92	4.22	5.14	4.65	5.62	4.91

* Cows that faced a decrease in progesterone levels in milk and blood during the estrus cycle.

Estrus detection criteria were the estrogen levels in milk and blood plasma, which according to the studies, was below 20 pg/ml in non-estrus cows and above 20 pg/ml in estrus cows. The results of estrogen measurement in milk and blood plasma of the studied cows have been presented in Table 2.

Table 2: Estradiol measurement in milk and blood plasma using ELISA method on days 0, 19, 20, 22, and 24 following the artificial insemination in picograms per milliliters

Ear tag Number	**Estrus cycle 0 days**		**Estrus cycle 19 days**		**Estrus cycle 20 days**		**Estrus cycle 22 days**		**Estrus cycle 24 days**	
1	**Milk**	**Plasma**	**Milk**	**Plasma**	**Milk**	**Plasma**	**Milk**	**Plasma**	**Milk**	**Plasma**
	41	62	7	17	6	11	2	9	5	12
2	48	59	5	17	4	8	5	9	4	11
3	35	57	8	19	7	13	7	11	6	10
*4	38	63	5	18	34	59	11	18	5	11
5	31	71	6	14	4	10	3	8	3	9
6	47	67	7	18	3	11	2	7	2	6
7	29	49	6	17	4	10	3	8	3	7
8	48	58	7	16	3	11	2	7	3	6
*9	35	72	8	14	39	63	9	16	5	11
10	38	69	9	14	6	10	3	9	2	9
11	42	68	7	15	7	12	4	8	3	7
12	40	71	6	18	7	10	3	8	3	6
13	42	55	9	18	9	14	9	12	8	9
*14	36	59	7	15	37	68	8	18	5	12
15	32	62	7	14	6	12	6	7	4	6
16	33	68	9	18	6	10	2	9	3	7
17	31	48	8	17	6	11	3	8	1	8
18	31	56	9	19	8	18	9	14	9	12
19	37	58	7	14	4	10	2	5	1	5

*20	38	63	9	19	36	67	9	12	6	14
*21	32	61	8	18	39	63	9	19	5	9
22	28	43	9	19	10	16	9	14	7	11
23	39	51	7	17	4	9	3	8	1	5
*24	38	58	9	20	31	61	10	13	5	12
25	41	64	6	21	6	10	5	9	4	9
26	38	63	7	18	8	9	4	8	3	6
*27	37	58	9	19	39	63	9	12	4	8
28	32	61	6	14	5	9	4	7	3	6
29	32	67	9	18	6	11	3	9	1	6
30	35	64	7	14	4	10	4	9	2	5

* Cows that faced an increase in estrogen levels in blood and milk during the estrus cycle

P4 test method:

Milking of the cows was done during evening milking time according to the hygiene guidelines. After milking of each cow, the milk was mixed and a 25 cc sample was taken, of which 5 cc was transferred to the kit tube, and the ear tag number of the cow was labeled on the tube kit. In addition, 20 cc of the sample was transferred to the sampling tube and was sent to the laboratory for being tested for progesterone and estrogen (Tables 3 and 4).

Table 3: The results from the P4 test on days 0 (estrus), 19, 20, 22, and 24 following the artificial insemination

Cow number	results from P4 test on days 0 (estrus), 19, 20, 22, and 24 following the artificial insemination					Pregnancy test by ultrasound on day 30	Pregnancy test by rectal examination on day 45
	Day 0 (heat) of the estrus cycle	Day 19 of the estrus cycle	Day 20 of the estrus cycle	Day 22 of the estrus cycle	Day 24 of the estrus cycle		
1	-	+	+	+	+	+	+
2	-	+	+	+	+	+	+
3	-	+	+	+	+	+	+
*4	-	-	-	-	-	-	-
5	-	+	+	+	+	+	+
6	-	+	+	+	+	+	+
7	-	+	+	+	+	+	+
8	-	+	+	+	+	+	+
*9	-	-	-	-	-	-	-
10	-	+	+	+	+	+	+
11	-	+	+	+	+	+	+
12	-	+	+	+	+	+	+
**13	-	+	+	+	+	-	-

*14	-	-	-	-	-	-	-
15	-	+	+	+	+	+	+
16	-	+	+	+	+	+	+
17	-	+	+	+	+	+	+
**18	-	+	+	+	+	-	-
19	-	+	+	+	+	+	+
*20	-	-	-	-	-	-	-
*21	-	-	-	-	-	-	-
**22	-	+	+	+	+	-	-
23	-	+	+	+	+	+	+
*24	-	-	-	-	-	-	-
25	-	+	+	+	+	+	+
26	-	+	+	+	+	+	+
*27	-	-	-	-	-	-	-
28	-	+	+	+	+	+	+
29	-	+	+	+	+	+	+
30	-	+	+	+	+	+	+

* Cows with low progesterone levels in milk by p4 kits and showed estrus again.

** Cows that were identified as non-pregnant by p4 kit despite the high level of progesterone in their milk.

Table 4: Accuracy of pregnancy detectionusing P4 kit

Pregnancy Status	Pregnant Cows	Non-pregnant Cows
True	23	7
False	3	-
% P.R	87%	100%
Total accuracy	93.5	

Results and Discussion

Determining progesterone levels in blood plasma and milk:

During this study, out of 30 inseminated cows, 7 had blood plasma and milk progesterone levels of less than 2 ng/ml. It should be noted that the Rapid P4 test results showed the progesterone levels low as well. After that, it was found that all 7 cows showed estrus, which made the accuracy of this kit for detecting estrus 100%. However, in 23 other cows which their progesterone levels were shown high by the Rapid P4 kit, it was found that the P4 kit detected 87% of the cases as true pregnancies and 13% as false pregnancies. Cows with true pregnancies had a progesterone concentration above 2 ng/ml. It can be stated that this hormonal evaluation was consistent with the findings of Stabenfeldt et al. (1969), Henricks et al. (1969) and Donaldson et al. (1970).

The detected false pregnancies by P4 test can be due to the long life of corpus luteum or early death of the embryo, which was consistent with the reports of Mekonnin et al. (2017). Mekonnin et al. (2017) stated that identification of reproductive status in cattle including estrus, pregnancy, the early death of the embryo and ovary issues were important in assessing fertility efficiency. The analysis of progesterone and estrogen in milk and blood could be used to determine all these conditions (Mekonnin et al., 2017). Suthanthirakannan et al. (2014) showed that heat cows had a

progesterone concentration of less than 1 ng/ml, which was in line with the previous studies on plasma serum (Alam et al., 1994) and milk (Moore et al. 1991). In another study, Nebel et al. (1988) concluded that the high levels of P4 clearly confirmed that the animal was not heat, even if it showed symptoms of estrus. Mekonnin et al. (2017) reported that the cyclic activity of the ovaries could be determined by sampling within an 11-day period so that two samplings of which, at least one of them, showed P4 to be more than 1 ng/ml indicating that the corpus luteum existed in the ovary. In this study, it was found that milk concentrations of P4 were closely associated with their blood levels. During the reproductive cycle of cattle, the only problem with milk sampling was the lactation cycle, so that dry cows and heifers could not be used as the subject of sampling.

Evaluation of estrogen levels in blood plasma and milk:

In this study, the maximum estrogen of milk was evaluated to be 48 pg/ml in the heat day, which was decreased below 10 pg/ml the day after the estrus. In addition, the maximum estrogen level in blood plasma was evaluated to be 72 pg/ml in the estrus day, which was decreased below 20 pg/ml the day after the heat. In this study, the range of estrogen on oestrus days was evaluated to be 30 to 70 pg/ml. In a previous study by Robertson (1969) the estrogen levels in the blood plasma of cows were evaluated to be in the range of 1 to 12 pg/ml. In humans, the average level of estradiol B_{17} was assessed to be 125 pg/ml using protein binding method and radioimmunoassay method evaluated it to be 108 pg/ml. (Roelofs et al., 2005). During the study of Scaramuzzi et al. (1970) on ewes, it was found that the concentration of ß17 estradiol in the ovarian vein can only be detected 2-3 days before estrus using fluorescence method, while it was detectable in the entire estrus cycle using radioimmunoassay. In addition, ß17 and steriol have been found in the urine of cows as well. The estrogen level of the current study was evaluated in a range from undetectable to 30 pg/ml, which was similar to the biology levels of 1-10 pg/ml. In this study, it was found that plasma estrogen was at the lowest level 4 days prior to heat, and the increase in estrogen may be due to follicular growth in the following days. High levels of estradiol were seen in cows showing heat in this study, which was consistent with other studies (Opara et al., 2006; Perry et al., 2014). Basically, it can be argued that determining the levels of steroid hormones using ELISA or commercial kits can be used to investigate the reproductive status of dairy cows. Using this method, the estrus cycle could be detencted in livestock and a successful insemination program could be performed, as well as pregnancy could be diagnosed as soon as 19 to 22 days following the insemination.

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