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Research Article

Lateral Flow Immunoassay for Whole Blood Progesterone Detection as a Tool for Assessment of Reproductive Status in Cattle

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ABSTRACT

Reproductive inefficiency is one of major challenges affecting dairy farming in Kenya. This is partly caused by poor estrus detection, delayed determination of unsuccessful artificial insemination (AI) and sub-optimal calving to conception interval. Blood levels of progesterone hormone (P4) are a valid indicator of the reproductive status of an animal. Progesterone levels can be used to determine the estrous phase of an animal and also pregnancy status. This study evaluated P4 levels across the reproductive cycle in dairy cattle in Kenya using the whole blood semi quantitative lateral flow immunoassay (LFIA) manufactured by Diagnostics for All Company and compared the findings with those by plasma quantitative enzyme linked immunosorbent assay (ELISA). Blood was collected from 46 animals to establish the P4 profiles at the various stages of the reproductive cycle using ELISA. Subsequently, P4 levels were analyzed by LFIA and ELISA in blood from 100 dairy cattle and the tests compared. The P4 profiles of dairy cattle in the study ranged from 0-10ng/ml. The LFIA test results were scored from 1-3. Progesterone levels with scores of 1 to 2 were recorded in pre-pubertal and follicular phase animals, corresponding to P4 levels ranging from 0.01 to 2.4 ng/ml by ELISA. Non pregnant luteal phase and pregnant animals had higher ($P>0.05$) LFIA scores of 3 representative of P4 levels ranging from 6 to 10 ng/ml. The semi-quantitative P4 levels as determined by the LFIA were highly correlated ($r = 0.95$; Kappa 0.93) with the quantitative ELISA P4 levels for low and high P4 concentration. The findings of the current study show that LFIA is a reliable method for determination of P4 levels in whole blood that can be used as a point of care decision support tool for reproductive management of dairy cows.

Key words: Cow, Progesterone, ELISA, LFIA

INTRODUCTION

Kenya is an agricultural economy based primarily on small-scale, resource-poor farmers, more than half of whom subsist on less than one US dollar per day. Livestock makes a significant contribution to the agricultural gross domestic product, with the dairy sub-sector supporting the livelihoods of more than one million people. Over 70% of the dairy output in the country is from cattle, of which more than 80% are reared by small-scale farmers (FAO, 2011). This small holder production system largely consists of zero grazing where 1 to 3 animals are confined in limited space. This affects the ability of animals to express overt signs of estrus, and when they do, the signs may not be noticeable. Since artificial insemination (AI) is the main mode of breeding the dairy cattle, accuracy and efficiency of estrus detection causes a challenge to attainment of optimum reproductive efficiency in small holder dairy farms.

Calving interval (CI) is the reproductive index commonly used to assess reproductive efficiency in dairy farms (French and Nebel, 2003). The CI is affected by calving to conception interval and the gestation period. Since the gestation period is fixed, the calving to conception interval is the critical variable and is influenced by the time to post-partum resumption of ovarian cyclicity, the occurrence and detection of estrus, and fertility at service. Accurate determination of estrus is therefore central to optimization of reproductive efficiency, especially where AI is used. Proper timing of AI influences fertility at service and is determined by when the animal was confirmed to have been seen on heat. Additionally, early determination of whether AI was successful or not would inform decision support for remedial action to target the recommended CI of 12-13 months. Various heat detection aids can be used to enhance the accuracy and efficiency of heat detection on dairy farms to enhance reproductive performance of the

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animals (Dalton, 2011). However, most of these may not be practical in small holder dairy systems, besides being unaffordable by the resource-poor farmers.

On farm cost-effective diagnostic tools to enhance estrus detection, inform appropriate timing of AI and indicate in good time the success or failure of an insemination would be useful in reproductive management of cows on small holder dairy farms. Progesterone detection kits can reduce reproductive inefficiency through increased heat detection rates, early pregnancy diagnosis and detection of infertility in the herd. Progesterone levels are low during proestrus and estrus and are elevated during diestrus and pregnancy (Purohit, 2010). Available methods for P4 analysis are time consuming, require laboratory facilities, and may not be affordable to resource-poor farmers (Safronova *et al*., 2012).

The Lateral flow assay (LFIA) is a simple, rapid, cost-effective diagnostic test that can be used to measure P4 levels in whole blood (Posthuma-Trumpie *et al*., 2009; Waldmann and Raud, 2016) for decision support in reproductive management of dairy cattle for improved productivity.

The aim of the present study was to evaluate the P4 profiles of dairy cattle in Kenya and also evaluate the efficiency of the semi quantitative LFIA in determination of P4 levels in whole blood in dairy cattle.

MATERIALS AND METHODS

Selection of animals

The study was carried out at the University of Nairobi dairy farm in Kenya, located on a 375 acre piece of land in Kiambu County, 15 kilometers west of Nairobi city. The dairy cattle breeds kept were Friesian, Ayrshire, Jersey, Guernsey and their crosses. The animals were reared in an extensive system where they grazed all day and supplementation was done with concentrates (dairy meal) and silage in the morning and evening. Water and mineral licks were provided ad libitum. Animals on the farm were bred by artificial insemination.

Records of 180 animals were examined. From this, 160 animals ranging from 5 months to 12 years of age were included based on their health status and reproductive history. The animals were categorized as pregnant or non pregnant. Further stratification of the pregnant animals was done based on the age of pregnancy as calculated from the date of insemination as first, second and third trimester of pregnancy. Based on age, non-pregnant animals were categorized as either pre-pubertal or pubertal. The subject pregnant and non-pregnant animals for the study were selected based on the reproductive status by generation of random computer numbers. The different breeds of cattle were represented in the sample size.

The reproductive status of the selected animals was confirmed by rectal palpation and trans- rectal ultrasonography. Ultrasonography was performed on non pregnant pubertal cattle and those that were expected to be 3 months and below pregnant. Trans-rectal palpation was done to confirm pregnancies that were 4 months and above. Positive diagnoses of pregnancy by trans-rectal ultrasonography were dependent on the detection of anechoic fetal fluids and/or the embryo proper in the uterine lumen (Lucy *et al.*, 2011). The ovaries were scanned for presence of the corpus luteum and/or follicles in non pregnant animals. The selected animals were then divided into two groups based on the theoretical expectation of high and low blood P4 levels. The high P4 group was composed of pregnant and non pregnant luteal phase and low blood P4 group was composed of non pregnant follicular phase and pre-pubertal animals. A third group of animals whose reproductive status was not confirmed by either rectal palpation or ultrasonography were also included in the study. A field evaluation form with details of the animal biodata, reproductive history, feeding regime, body condition score and bodyweight of each selected animal was filled. The body condition score was obtained using a 5 scale grade according to Edmonson *et al*. (1989) and bodyweight in Kilograms taken using a weighing band (Dingwell *et al.*, 2006). The animal protocols used were approved by the Biosafety, Animal use and care committee Faculty of Veterinary Medicine, University of Nairobi.

Blood collection and sample handling

Cumulatively, 146 blood samples were collected from the herd. Initially, 46 samples were obtained to determine plasma P4 levels during the reproductive cycle by ELISA and later 100 samples collected for determination of P4 levels in whole blood by LFIA and in plasma by ELISA. The animals were physically restrained in a chute and 10 ml of blood collected from the coccygeal vein into heparinized tubes labelled with the animal identification number and date of collection. The blood samples were stored in a cool box filled with icepacks. In the first phase, blood samples were transported to the laboratory immediately after collection but in the second phase, a pilot study of the lateral flow assay was carried out in the field after blood collection and transportation of the remaining blood samples to the laboratory was done afterwards within 2 hours of collection. In the laboratory, centrifugation of a portion of heparinized blood was done at 1000-2000 x g for 15 minutes to obtain plasma. The remaining portion of heparinized blood was used for lateral flow test in the laboratory. The plasma was harvested into eppendorf tubes and plasma ELISA was run side by side with the lateral flow assay.

Progesterone analysis by LFIA

The lateral flow assay for each sample was done in triplicate. The procedure was done according to the manufacturer's instructions, the Diagnostics For All Company. Briefly, a drop of heparinized blood (35µl) was pippeted into the sample well and 35 µl of diluent added. In a second assay well, 75 µl of chase buffer was added. The LFIA strip was then inserted into the sample well that contained the blood and diluent and incubated for 5 minutes, and then moved to the second well with the chase buffer for 10 minutes. After incubation in the chase buffer, the strip was removed from the assay well and results which were in the form of color development at the test line on the strip interpreted using the read guide chart. The guide chart (Figure 1) was a sheet of paper made up of three columns of LFIA strips with different reference

color intensities of the test lines. The three columns were labelled as score 1, 2 and 3 in which column one (score 1) consisted of reference LFIA strip test lines with high color intensity (dark red). Column two (score 2) consisted of the reference LFIA strip test lines with a medium color intensity (faint red color) and Column 3 (score 3) was made up of reference LFIA strip test lines with very low color intensity to a non visible test line. According to the manufacturer, score 1 corresponded to low P4 levels (0-3ng/ml), score 2 mid P4 levels (4-6ng/ml) and score of 3 was interpreted as high P4 concentration (7-10ng/ml).

Scoring of the LFIA results was done visually with the naked eye by final year Veterinary Medicine students at the University who were not aware of the status of the subject animals. Lateral flow assay strip images were digitized by scanning using Doxie flip scanner (Apparent Corporation, 121 Dry Ave, Cary, NC 27511 USA) and quantification in optical units of the color intensity of both the test and control lines of the digital images done by a software image analysis program (Image j: https://imagej.nih.gov/ij/)), so as to obtain true intensity of the color development of the test line. The intensity for each individual strip of each sample was obtained and an average of the intensity of the three strips per sample was calculated. The overall intensity of the LFIA strips in animals at different stages of the reproductive cycle was obtained. The obtained overall mean intensity for each category of animals was used to draw graphs against the corresponding lateral flow assay strip scores and also corresponding quantitative P4 levels by ELISA.

Progesterone analysis by ELISA

Progesterone analysis was done using ELISA (Ovucheck®, Biovet) as described by Samsonova *et al*. (2015). Briefly, aliquots of 10µl of standard solutions, controls and samples were added to appropriate micro plate wells followed by 200µl of conjugate. After incubation for 30 minutes and washing, 200µl of substrate was added to each well. The color reaction was stopped after 30 minutes incubation with 100µl stop solution. The results were evaluated on the ELISA plate reader at 405-450nm wavelength. Standard curve was drawn using the optical density values for the standards as Y values and the corresponding P4 concentration as the X values and the equation for calculation of the corresponding P4 levels of the samples was derived from the standard curve. The intra assay coefficient of variation for the ELISA was 7.5% and inter-assay coefficient of variation was 15%.The calibration curve of the ELISA ranged from 0ng/ml to 10 ng/ml.

Statistical analyses

The data was analyzed using the Stata statistical software (Version 12, College station, TX: StataCorp LP). Significant differences in mean P4 levels in animals at different stages of the reproductive cycle was obtained by Analysis of Variance (ANOVA) and student t test statistics. Statistical significance was set at probability values of < 0.05.

The diagnostic parameters of LFIA test were calculated based on formulas by Karen *et al*., (2015) and Martin *et al*., (1987). The LFIA results were classified as either correct positive (a), false negatives (b), or false positives (c), correct negatives (d). The following diagnostic parameters were calculated: Sensitivity [(a/a + b) x 100], specificity [(d/c + d) x 100], positive predictive value [(a/a + c) x 100], negative predictive value [(d/b+ d) x 100], overall accuracy [(a + d/a + b + c + d) x 100]. A 95% confidence interval of each accuracy parameter of the diagnostic tests was determined.

Sensitivity was defined as the ability of the LFIA test to correctly detect low P4 (positives) as the same as ELISA does. Specificity was defined as the ability of the test to correctly identify high P4 animals (negatives) determined to have high P4 by ELISA. The positive predictive value (PV+) was the probability of a positive diagnosis by the LFIA test being further corroborated by the ELISA. The negative predictive value (PV-) was the probability of negative results by the LFIA test being corroborated the ELISA results. Accuracy was defined as the ability of the LFIA test to correctly diagnose high and low P4 cows among those diagnosed as high and low by ELISA test.

The correlation coefficient (r) and Kappa statistics were used to assess the agreement between the LFIA strip scores (semi quantitative analysis) and quantitative analysis of P4 (Martin *et al*., 1987).

RESULTS

Study animals

From the records of 180 female animals, 160 of them met the inclusion criteria of being aged between 5 months to 12 years, had good reproductive history and health status. There were 40 pregnant and 120 non pregnant animals. Of the pregnant animals, based on insemination records, 12, 18 and 10, were categorized to be in the first, second, and third trimesters of pregnancy, respectively. Forty two of the non pregnant animals were pre pubertal, whereas 78 were pubertal. Pregnant and non-pregnant animals were randomly selected and the reproductive status confirmed by ultrasonography and rectal palpation. From the confirmation, 30 randomly selected pregnant animals were grouped as the high P4 group. For the 30 randomly selected pubertal animals, 14 in the follicular phase, 2 in estrus, 5 one week postpartum, and 8 that were one day post insemination were assigned to the low P4 group. Forty one animals were selected from the remaining number of animals and their reproductive status was not confirmed by neither ultrasonography nor rectal palpation before sampling. These consisted of the unknown group. All the four breeds (Friesian, Ayrshire, Jersey, Guernsey and their crosses) were represented based on the proportion of animals of the individual breed in the herd.

After sampling and blood P4 analysis, the animals were re-grouped in different stages of the reproductive cycle based on the concentration of P4, ultrasound, rectal palpation status and reproductive status from the records. The groups were as follows: 35 pregnant (4, 15, and 16, in the first, second, and third trimester, respectively), 17 non pregnant luteal phase, 14 follicular phase, 2 in estrus, 5 one day post insemination and 19 pre-pubertal animals.

The animals had a median body condition score of 3 on a scale of 1 to 5, where 1 was emaciated and 5 was obese animals. The bodyweight ranged between 167 Kilograms for pre-pubertal heifers to 600 Kilograms for cows with an average of 380 ±12.88 Kilograms.

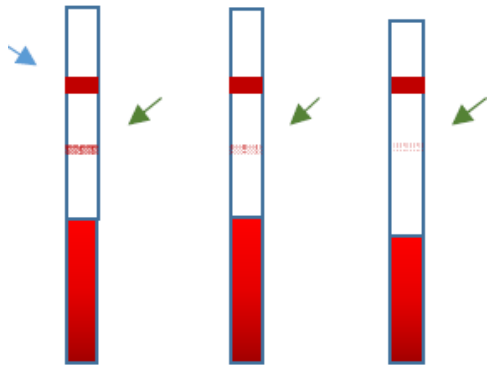

Fig. 1: Reference red guide chart

LFIA strips results

The pregnant animals in the first trimester had a mean strip score of 3±0, second trimester had a mean score of 2.997±0.1066 and third trimester had a mean strip score of 3±0. The pregnant animals had a mean LFIA score of 3. The pre-pubertal heifers less than 13 months had a mean strip score of 1.64±0.47, while day one post-insemination animals a mean score of 1.3±0.70 and lastly, one week postpartum animals had a mean LFIA score of 1.31± 0.45 (Figure 2).

The LFIA scores of low P4 animals (prepubertal heifers, one week postpartum and day one post insemination) were similar (P>0.05). Additionally, there was also a similarity in the LFIA scores between the first, second and third trimester of pregnancy (P>0.05). LFIA scores of the high P4 animals (pregnant animals) was higher (P<0.05) than that of low P4 animals (day one post insemination, one week postpartum, prepubertal heifers (Figure 2).

Colour intensity of the LFIA strips as analyzed by image j software

The color intensity of the strips as analyzed by Image j software is shown in Figure 3. High intensity of the LFIA strip testlines corresponded to low P4 levels while low intensity of the LFIA strip testlines corresponded to high P4 levels. The high intensity in optical units was recorded in pre-pubertal heifers, day one post-insemination and one week postpartum whereas the low intensity in optical units was recorded in pregnant animals (Figure 3).

A similarity of the intensity values of pregnant animals across the first, second and third trimester of pregnancy was recorded (P>0.05). There was also a similarity in the intensity values of low P4 animals (pre-pubertal heifers, day 1 post-insemination and one week postpartum; P>0.05). The intensity values for the high P4 animals (pregnant animals) was higher (P<0.05) than that of low P4 animals (pre-pubertal heifers, day 1 post-insemination and one week postpartum animals).

Comparison of the color intensity of LFIA strips with the LFIA scores

There was a significant difference in the LFIA strips test-lines intensity of high and low P4 levels. There was a similarity between the intensity of strips test-line with intensity of 1 and 1.5. Scores of 2, 2.5 and 3 were significantly different (Figure 4).

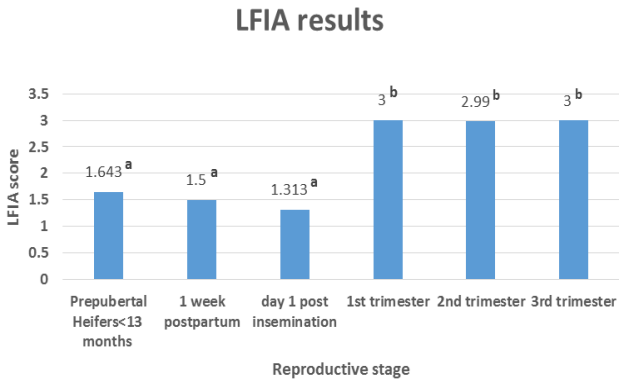

Fig. 2: Mean LFIA strip scores at different stages of the reproductive cycle. Bars with different superscripts have significantly different LFIA scores.

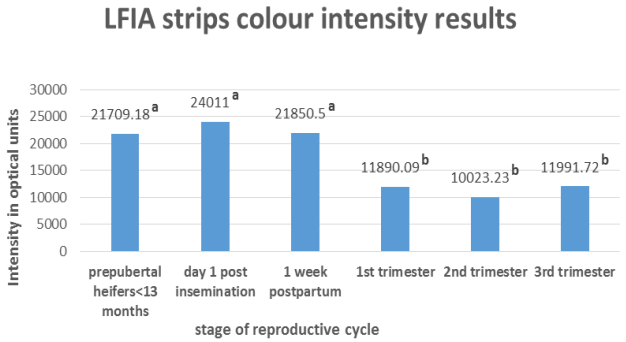

Fig. 3: LFIA strip colour intensity at different stages of the reproductive cycle. Bars with different superscripts have significantly different intensities.

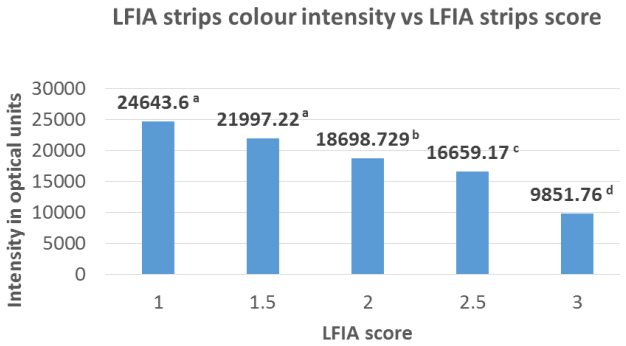

Fig. 4: Intensity of LFIA strip testline against LFIA scores. Bars with different superscripts have significantly different intensities.

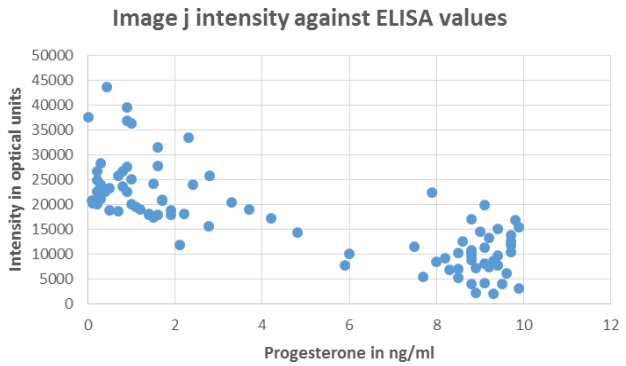

Fig. 5: Color intensity of the LFIA strips against P4 levels determined by ELISA.

Table 1: Progesterone levels of cattle across the reproductive cycle by ELISA and LFIA

Sample size	Category of animals	LFIA score in the lab	Image j LFIA strips intensity	Mean P4 levels by ELISA (ng/ml)
19	Pre-pubertal heifers 13 months and below	1.6±0.47[a]	21709.18±7139.60[a]	1.453±0.950[a]
14	Cows in follicular phase	1.25±0.31[a]	22931±6578.25[a]	1.234±1.0623[a]
2	Estrus	1.0[a]	27493.67±1123[b]	0.3046±0.151[a]
5	One week postpartum	1.3±0.45[a]	21850.5±5569.88[a]	1.066±0.5242[a]
8	One day post insemination after estrus synchronization	1.3±0.70[a]	24011±8965.98[c]	1.041±0.642[a]
17	Non pregnant luteal phase	3.0[b]	11952.23±2034[d]	7.972±1.852[b]
35	Pregnant	3.0[b]	11301.67±4834.69[d]	8.876±0.7823[c]

Values with different superscripts within the same column differ significantly (P<0.05).

Table 2: Diagnostic values for the LFIA test

	LFIA positive (score 1-2)	LFIA negative (score 3)	Total
ELISA positive	49	1	50
ELISA negative	4	46	50
Total	53	47	100

Specificity	0.92	46/50	Confidence interval (0.87-0.97)
Sensitivity	0.98	49/50	(0.95-1.00)
PV-	0.98	46/47	(0.95-1.00)
PV+	0.92	49/53	(0.87-0.98)
Accuracy	0.95	46+49/100	(0.92-0.99)

The comparison of LFIA and ELISA results were strongly correlated with a correlation coefficient of 0.95 and Kappa agreement of 0.93.

Blood P4 levels as determined by LFIA and ELISA

Mean P4 levels across the reproductive cycle by both ELISA and LFIA are shown in Table 1. The pre-pubertal heifers aged 13 months and below, animals in estrus, day one post insemination animals, one week postpartum animals and follicular phase animals had low P4 levels with an LFIA score of less than 2 which corresponded to high color intensity of the test-line. Pregnant and non pregnant luteal phase animals had LFIA average strips scores of 3, low color intensity of the test line with high mean P4 levels.

The LFIA score and color intensity of the test line of pregnant and non-pregnant animals in luteal phase were similar (P>0.05), however there was a slight statistical difference in the means of ELISA P4 levels ng/ml of the two groups (P<0.05). The LFIA scores and test line color intensity for luteal phase and follicular phase non pregnant animals were different as well as the mean P4 levels in ng/ml (P<0.05). The mean P4 levels by both LFIA, image j test line intensity and ELISA for the low P4 animals (pre-pubertal heifers, cows in follicular phase, one week postpartum cows and day one post insemination) were significantly different (P<0.05) from those of high P4 animals (pregnant and non-pregnant luteal phase).

Breed, body weight and body condition score did not have a significant effect on the levels of P4 both in ELISA and LFIA test in animals at the same stage of reproductive cycle (P>0.05).

Color intensity development of the LFIA strips test and P4 levels as determined by ELISA

High color intensity values of the LFIA test lines of above 18000 corresponded to low P4 levels of 4 ng/ml and below whereas low color intensity of LFIA test lines values of below 12000 corresponded to high P4 levels of 6ng/ml and above as shown in Figure 5.

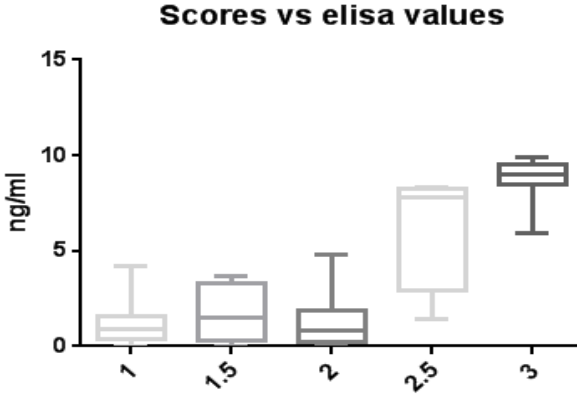

Fig. 6: Quantitative P4 levels in ng/ml against the corresponding LFIA scores.

LFIA score with corresponding P4 levels by ELISA in ng/ml

LFIA score from 1 to 1.5 had corresponding mean ELISA P4 levels of below 4 ng/ml. Most values with a score of 2 had P4 levels of below 3ng/ml, whereas others had corresponding P4 levels of between 4-4.5 ng/ml. A score of 2.5 corresponded to P4 levels of above 4 ng/ml though few lower limit values corresponded to P4 levels of 2 ng/ml. The score of 3 had corresponding mean ELISA P4 levels of 6ng/ml and above (Figure 6).

Calculation of diagnostic values of LFIA

The LFIA test diagnostic values sensitivity, specificity, predictive values and accuracy were calculated with the ELISA results as the gold standard (Table 2).

DISCUSSION

Progesterone profiles were determined in the current study by ELISA in Kenyan dairy cattle and the concentrations were similar to those reported in other studies elsewhere (Osman *et al*., 2012; Naik *et al*., 2013). Additionally, lateral flow immunoassay was used to determine the P4 levels in whole blood in cattle at different stages of the reproductive cycle. This is the first documentation of use of whole blood for P4 determination by LFIA in the bovine.

Lateral flow immuno assays are qualitative, semi quantitative and quantitative tests that can be used in non-laboratory environments which are similar to the field conditions found in dairy production systems in Kenya. The LFIAs have been used for various diagnostic purposes (detection of hormones, drugs, pathogens and metabolites in biomedical, phytosanitary, veterinary, feed/food and environmental settings) including

determination of P4 in milk to assess estrus and pregnancy status in dairy cattle (Posthuma-Trumpie *et al*., 2009; Samsonova *et al*., 2015; Waldmann and Raud, 2016). Information on use of LFIAs in assessment of circulating P4 levels in blood in dairy cattle, and especially in whole blood in dairy cattle is scarce. Progesterone levels were determined in the current study in whole blood across various reproductive phases in dairy cattle in Kenya, and the values compared with those detected at the same time in the same sample by ELISA.

Using LFIAs, determination of P4 levels in a sample is based on the color intensity of the test line on the LFIA strip, and the amount of P4 in the sample is inversely proportional to the color intensity of the test line. Low P4 levels are expected in pre-pubertal, follicular phase, estrus, one week postpartum and day one post-insemination animals (Nebel *et al*., 1987; Safronova *et al*., 2012), since their ovary is devoid of the corpus luteam which is the main source of P4 in non pregnant animals (Nebel *et al*., 1987; Rioux and Rajotte, 2004; Cooke and Arthington, 2009). In the current study, these categories of animals corresponded to a high color intensity (score 1-2) of the test-lines on the LFIA strips, indicative of low P4 levels. The findings were in agreement with those of Waldmann and Raud (2016) and Samsonova *et al*. (2015) who reported highest intensity of the LFIA test line for low P4 in milk for animals in estrus or follicular phase. The LFIA strip color intensity was low (score 3), indicative of high P4 levels, in luteal phase and pregnant animals, as is expected physiologically (Nebel *et al*., 1987; Safronova *et al*., 2012). Waldmann and Raud, (2016) also reported lowest color intensity of the test line to almost non visible in milk in high P4 animals. Samsonova *et al*. (2015) also recorded low intensity of the test line in pregnant animals. These animals have high progesterone levels since they have a functional corpus luteum and a placenta which is an additional source of P4 for pregnant animals (Nebel *et al*., 1987; Safronova *et al*., 2012). The LFIA is reliable for determination of P4 in whole blood as it was in milk.The use of whole blood enables assessment of P4 levels in animals at all stages of the reproductive cycle whereas use of milk is restricted to lactating animals only.

The P4 levels as indicated by the LFIA strip visual scores and test-lines color intensities were compared with quantitative P4 values as determined by ELISA. There was a high concordance in LFIA and ELISA P4 findings (r 0.95; kappa 0.93) with scores 1 to 2 representing low P4 levels and scores 3 indicating high P4 levels. These results indicate that LFIA is reliable in detection of P4 levels in whole blood. Additionally, the high statistical difference between the LFIA scores (1-2 and 3; $P<0.05$), further affirms that the LFIA can be used to determine P4 levels in whole blood in dairy cattle at various phases of the reproductive cycle. The sensitivity of the LFIA was 98% with an accuracy of 95%. The Kappa agreement between the LFIA and ELISA was 0.93 with a correlation coefficient of 0.95. The specificity of the LFIA test was 92%. These LFIA diagnostic parameter values compare favorably with those obtained previously using milk for P4 determination by LFIA (Samsonova *et al.,* 2015; Waldmann and Raud, 2016). Lateral flow immunoassay is therefore reliable in determination of P4 levels in whole blood during different phases of the reproductive cycle in dairy cattle.

There was a discrepancy in the P4 levels by ELISA in samples with LFIA scores of 2 and the manufactures guidelines of expected P4 levels at the LFIA score of 2 in this study. According to the manufacturer, score of 2 should correspond to P4 levels between 4-6 ng/ml. However, in the current study those samples that were recorded to have LFIA scores of 2 had P4 levels of 2-4 ng/ml and high color intensity of the test line affirming that indeed the P4 levels were low. It was therefore, concluded that there could have been a human error in the reading of the visual card for the score of 2. The error could possibly have occurred due to the fact that the visual color intensity of last reference test-line of the score 1 column on the read guide chart was almost similar to that of the first reference test line in the score 2 column. Therefore, due to subjectivity of the naked eye visualization, some people would score it as 2 and others as 1. Therefore, the authors of this paper, recommend that the manufacturer of the LFIA kit should adjust the read guide chart reference test lines to ensure ease of visual differential across the scores.

Progesterone levels as determined in the current study by ELISA were higher in the pregnant animals (8.876 ± 0.7823ng/ml) compared to the luteal phase non-pregnant animals (7.972±1.852 ng/ml). However, the P4 levels as detected by the LFIA did not differ between the pregnant and non-pregnant luteal phase animals (score 3). Although an explanation for these ELISA findings was not available, the LFIA findings in the current study are supported by those of Ghanem and Nishibori, (2015) who reported similarity in ELISA determined P4 levels of pregnant and non-pregnant cows with normal luteal function. The most advanced stage of pregnancy in the animals in the current study was 230 days. The P4 levels among the pregnant animals ranged from 7.847 ng/ml to 10.493 ng/ml. Although not statistically different, more advanced pregnancies had higher P4 levels than the early pregnancies. Probably had the sample size been bigger, and more late pregnancy animals included in the sample such differences in P4 levels at different gestational stages may have been seen. This observation was similar to that of Mukasa-Mugerwa and Tegegne, (1989), who documented variations in ELISA determined P4 levels across the gestation period with a significant increase in P4 levels in the second and third trimester of pregnancy.

A limiting factor of the LFIA strip is that the scoring with the naked eye could be subjective. To address this the current study digitized the intensity of the test lines using a scanner and the intensity signals quantified by image software analyzer to obtain objective results. This confirmed the visual scoring, further supporting that LFIA is a reliable indicator of P4 in whole blood.

In conclusion, LFIA is a reliable, simple, rapid test that could be used at the point of care for determination of P4 levels in blood for decision support in reproductive management of dairy animals. In small holder dairy farms where estrus detection and timing of AI are a challenge, LFIA can be used to determine P4 levels to assess whether animals presented for AI are truly in estrus and also to detect the outcome of AI by determing the P4 levels early (when the animal is expected to be next in estrus) for corrective measures.

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