

Evaluation of synchronization protocols and methods of early pregnancy diagnosis in dairy cattle

A.H. Shahzad^{1#}, A. Sattar¹, N. Ahmad¹, I. Ahmad², M.S. Yousaf², S. Abbas², R.S. Blouch³, D. Nak⁴, Y. Nak⁴, D.L. Ray⁵ & W.J. Silvia⁵ (deceased)

¹Theriogenology Department, University of Veterinary and Animal Sciences, Lahore-Pakistan

²Department of Physiology, University of Veterinary and Animal Sciences, Lahore-Pakistan

³Livestock and Dairy Development Department, Punjab

⁴Department of Veterinary Obstetrics and, Gynecology Uludag University, Bursa-Turkey

⁵Department of Animal and Food Sciences, University of Kentucky, Lexington 40546-0215

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Abstract

The studies aimed to evaluate the pregnancy rate (PR) for timed artificial insemination (TAI) after G7G-Ovsynch, modified G7G-Ovsynch (MG7G-Ovsynch) and Ovsynch protocols and to assess the accuracy of using pregnancy-associated glycoproteins (PAGs) and plasma progesterone (P_4) in pregnancy diagnosis compared with ultrasonography (US). In study 1, Holstein cows ($n = 37$) were bred by TAI following the G7G-Ovsynch protocol ($n = 19$) or MG7G-Ovsynch ($n = 18$). Pregnancy was evaluated by US at days 31, 59, and 87 after breeding. The PR was not different for the G7G-Ovsynch and MG7G-Ovsynch. Blood and milk samples were collected on day 3 after insemination and then weekly through day 59 post TAI in cows diagnosed as not pregnant on day 31 and through day 87 in pregnant cows. PAGs were measured using ELISA and P_4 by radioimmunoassay (RIA). In the second study, Holstein cows ($n = 212$) were bred by TAI following G7G-Ovsynch protocol ($n = 110$) or standard Ovsynch ($n = 102$). Cows were subjected to pregnancy diagnosis on days 30, 60, and 90. A subset ($n = 15$ in each group) was subjected to blood and milk samples on days 30, 45, 60, 75, and 90 to measure PAGs and P_4 . In study 2, PR was not significantly different between synchronization protocols on days 30, 60, and 90. Pregnancy loss averaged 15% between day 30 and day 90. The use of PAGs and P_4 proved equally effective in diagnosis of pregnancy. Thus, G7G-Ovsynch was deemed the protocol of choice in postpartum cows, and PAGs assayed in milk or plasma could be used to diagnose pregnancy.

Keywords: artificial insemination, oestrous synchronization, Ovsynch, postpartum cow, pregnancy associated glycoproteins, ultrasonography

#Corresponding author: ahshahzad@uvas.edu.pk

Introduction

Reproductive efficiency is an important component of overall herd profitability. The factors responsible for efficient reproductive performance include herd health, nutrition, cow comfort, management decisions, manual labour, and improved reproductive management. Accurate heat detection, proper breeding methods and early pregnancy diagnosis (EPD) are instrumental in improving reproductive management. Failure to detect oestrus and improper breeding time, however, are the main constraints to reproductive efficiency. Several reproductive management protocols have been designed for planned breeding and implemented on large commercial dairy setups with mixed results.

Despite increased understanding of reproductive physiology, PR has decreased from 66% to 28% over the past five decades (Lucy *et al.*, 2001). Failure to detect oestrus in high-producing dairy cows necessitates TAI-based synchronization strategies. For this purpose, gonadotropin releasing hormone (GnRH)-based synchronization protocols are being utilized. These protocols facilitate TAI and eliminate the need for heat detection. A major limitation of these protocols is the lack of tight synchrony of ovulation (Colazo & Mapletoft, 2014). The introduction of the Ovsynch protocol by Pursley *et al.* (1995) is believed to be a landmark in controlled breeding. It consists of an injection of GnRH which causes ovulation

of a dominant follicle (if present). Seven days later, a 35 mg intramuscular injection of PGF₂α is administered to cause regression of any corpus lutea that is present. Two days later, the cows receive another injection of GnRH to cause ovulation of the new dominant follicle. Finally, 24 hours later the cows are artificially inseminated. As Ovsynch grew more universal, scientists appraised procedures to fine-tune it. The ovulatory response to the first injection of GnRH in the Ovsynch protocol was highly variable depending on the time it was administered during the oestrous cycle (Vasconcelos *et al.*, 1999). Based on these findings, many modifications were introduced (Bello *et al.*, 2006; Dirandeh *et al.*, 2015). Presynch protocols consisting of a single injection of PG followed by a GnRH injection two days later, and six or seven days before the standard Ovsynch (G6G or G7G, respectively) improved the PR following TAI (Bello *et al.*, 2006). The G7G-Ovsynch protocol seems to be more practical because most injections are given weekly.

Early pregnancy detection is needed for efficient management of the dairy because non-pregnant animals will need to be either synchronized again or culled. Rectal palpation (RP) and US are commonly used methods for EPD. With RP, pregnancy can be diagnosed at about day 35 post AI by a skilled person (Thompson *et al.*, 2010). Although RP is used widely, and is a simple and low cost method, it may cause untoward pregnancy termination. Ultrasonography is also useful for EPD, and can be used to assess foetal viability and sex. It is less invasive than RP, but requires sophisticated equipment and a degree of technical skill. Elevated P₄ concentration in serum or milk between 18 and 24 days post breeding is a valuable indicator of pregnancy, but requires knowing the exact breeding date. Pregnancy associated glycoproteins (PAGs) have been proposed as an alternative bio-marker for pregnancy (Sasser *et al.*, 1986). They can be detected following the third week after breeding in milk and blood.

The main objectives of this study were i) to compare G7G-Ovsynch with MG7G-Ovsynch and standard Ovsynch protocols in postpartum Holstein cows; ii) to evaluate pregnancy status with PAGs, plasma progesterone and US as tools for PD; and iii) to evaluate the effect of factors such as parity, P₄, foetal sex and sire effect on PAGs in milk and plasma. It was hypothesized that the G7G-Ovsynch protocol with two injections of PGF₂α will improve the PR and PAG-based pregnancy diagnosis as an early effective, skill-oriented cost-effective tool.

Materials and Methods

The Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky approved the protocol procedure (IACUC #2013-1153) of the current study.

The first experiment was conducted with Holstein cows (n=40) maintained at Coldstream Dairy Farm (Lexington, Kentucky, USA) (38° 06' 38.3" N 84° 30' 53.6" W) between August 2013 and February 2014 using multiparous (2.5 average lactations) lactating dairy cows. The cows were randomly assigned to two treatments with 20 cows in each. During the study, three cows were removed because of health issues, resulting in the groups having 19 and 18 cows. Cows were housed in free stall barns with one hour access to a pasture daily for exercise. They were fed a total mixed ration (TMR) once daily with ad libitum access to feed and water. Fans were provided in the holding pen and in the free stall barns, and sprinklers were installed above the feed bunk. The cows were milked twice daily (04h30 and 15h30) with the Afimilk (Kibbutz Afikim, Israel) milking system. They were fed a 17% crude protein-based TMR consisting of corn silage, alfalfa haylage, cottonseed, ground corn, soybean meal, distillers grains, and a mineral mix (with amounts of the ingredients varying slightly throughout the study) at 03h00 and 13h00. The first group received the G7G-Ovsynch protocol (day -2 prostaglandin F₂ alpha (PGF₂α; Lutalyse, Pfizer, New York, USA), day 0 GnRH (Factrel, Fort Dodge Animal Health, Collegeville, Pennsylvania, USA) day 7 GnRH, day 14 PGF₂α, day 16 GnRH) for oestrous synchronization. The second group received a modified treatment (MG7G-Ovsynch) with two injections of PGF₂α administered at eight-hour intervals on day 14. Insemination in both groups was done 20 hours after the final injection of GnRH. All cows were 45 to 90 days post partum at the start of the synchronization protocol and thus 65 to 110 days post partum at the time of AI. Pregnancy was determined using trans-rectal US at day 31 and reconfirmed at days 59 and 87 post AI.

Milk and blood samples were collected from all of the cows, starting three days after insemination and then every seven days. Sampling continued through day 59 post AI if the cow was not diagnosed pregnant on day 31 and through day 87 if the cow was pregnant on day 31. Milk samples (35 mL) from each cow were collected in plastic tubes, mixed with bronopol preservative and stored at 4 °C until they were shipped, for arrival the same day, to IDEXX Laboratories (Westbrook, Maine) to determine PAGs. Blood samples were collected by puncturing the median caudal vein with a syringe, with the blood being drawn into a vacuum tube and centrifuged at 2800x gravity for 20 minutes. After centrifugation, the plasma was transferred to two vials and frozen. One vial was shipped with the milk samples to IDEXX to determine PAG. The other vial was used to quantify P₄ using a solid-phase, RIA kit (Coat-a-Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA). The concentration of PAGs in plasma and milk samples was determined with ELISA kit procedures. Both the plasma and milk-based assays were designed to be qualitative, classifying

the results as pregnant and not pregnant. Reports from IDEXX included the qualitative assessment and optical density (OD).

In the second study, Holstein cows ($n = 212$) maintained at Akbaslar Dairy, Bursa, Turkey, were bred using fixed-time AI (FTAI). The synchronization protocols were G7G-OvSynch, as in the first study, and the conventional OvSynch protocol. Pregnancy was determined by transrectal US at 30 days after insemination and confirmed 60 and 90 days post AI. Starting on day 30 post AI, only 15 pregnant cows from each treatment were sampled at fortnightly intervals for PAG evaluation in plasma and milk. The procedures were similar to those used in the first study. After milking, the samples were refrigerated and a blood sample (10 ml) was collected. The PAG and P_4 concentrations were determined with an ELISA kit. Both the plasma and milk-based assays were designed to be qualitative, classifying the cows as pregnant or not pregnant.

The effects of treatments and parity on pregnancy rates were analysed with chi-square procedures in PROC FREQ of SAS (SAS Institute, Inc., Cary, North Carolina, USA). The effects of treatment on PAGs in milk and plasma and P_4 profiles in plasma were determined with the GLM procedure of SAS. The accuracy of the milk-based ELISA was determined by comparing the positive predictive values (PPV) and negative predictive values (NPV) of the assay to the established plasma-based assay. The sensitivity of the assay was evaluated by comparing the adjusted OD measurements of samples collected from pregnant and non-pregnant cows over time. Correlations of P_4 with the concentrations of PAGs in serum and milk were quantified using the REG procedure of SAS. Effects were declared significant at $P < 0.05$ and a trend towards significance was indicated when $P \leq 0.10$.

Results and Discussion

In the first study, the PR in the cows of the G7G-OvSynch group was 47% compared with 50% in the MG7G-OvSynch group at day 31 post AI ($P = 0.87$). At days 59 and 87 post AI, PR was recorded as 37% on both days in the G7G-OvSynch group, compared with slightly lower values (33%) in MG7G-OvSynch ($P = 0.82$). Overall pregnancy loss in the G7G-OvSynch group was 11%, whereas in MG7G-OvSynch it was 17% ($P = 0.59$) (Table 1). In the second study, PR in the G7G-OvSynch group was non-significantly ($P = 0.16$) higher (52%) compared with those of the OvSynch group (42%) on day 30 post AI. On day 60 post AI, PR was 45% in the G7G-OvSynch group compared with the OvSynch group, in which PR was 37% ($P = 0.23$). Pregnancy date on day 90 was 44% in the G7G-OvSynch group in comparison with 36% in the OvSynch group ($P = 0.28$). Overall pregnancy loss in the G7G-OvSynch group was 16% and in the OvSynch group it was 14% ($P = 0.80$) (Table 2).

Table 1 Pregnancy rates and losses in lactating dairy cows synchronized and inseminated by timed artificial insemination

Variable ¹	First experiment			Second experiment		
	G7G	MG7G	<i>P</i> -value	G7G	OvSynch	<i>P</i> -value
Pregnancy rate, 31 (30) days post AI	47% (9/19)	50% (9/18)	0.87	52% (57/110)	42% (43/102)	0.16
Pregnancy rate, 59 (60) days post AI	37% (7/19)	33% (6/18)	0.82	45% (50/110)	37% (38/102)	0.23
Pregnancy rate, 87 (90) days post AI	37% (7/19)	33% (6/18)	0.82	44% (48/110)	36% (37/102)	0.28
Pregnancy loss, 59 (60) days post AI	11% (2/19)	17% (3/18)	0.59	12% (7/57)	12% (5/43)	0.92
Pregnancy loss, 87 (90) days post AI	0% (0/19)	0% (0/18)	-----	4% (2/50)	3% (1/38)	0.72
Pregnancy loss, 31 (30) to 87 (60) days post AI	11% (2/19)	17% (3/18)	0.59	16% (9/57)	14% (6/43)	0.80

¹ Number of days given in parentheses is for the second experiment
AI: artificial insemination; TAI: timed artificial insemination

The animals that had been used in the first study were divided into two groups by parity. One group contained animals in their first and second parities ($n = 23$) and the second group contained animals in their third to fifth parities ($n = 14$). Pregnancy rates were significantly different between these groups (Table 2). Four pregnancies (57%) were lost between day 31 and day 59 in the cows in their first or second parity.

Table 2 Effects of parity on pregnancy rates following oestrous synchronization and timed artificial insemination

Rates	1st and 2nd parity	3rd to 5th parity	P- value
Pregnancy 31 days post AI	30% (7/23)	71% (10/14)	0.02
Pregnancy 59 days post AI	13% (3/23)	71% (10/14)	<0.01
Pregnancy 87 days post AI	13% (3/23)	71% (10/14)	<0.01
Calving rate	13% (3/23)	71% (10/14)	<0.01

In the first study, pregnancy was diagnosed with ELISA-based assays of PAGs from milk and plasma, plasma P₄ and trans-rectal US on days 31, 59, 87. The results of pregnancy diagnoses using milk and plasma PAGs and plasma P₄ were compared for those animals that were diagnosed pregnant by US (Table 3). Overall, 18 animals were pregnant on day 31 after AI. These results were considered 'gold standard' and compared with milk- and plasma-based PAGs and P₄ on days 31, 59, and 87 post FTAI (Table 4). In the ELISA-based PAG method, sensitivity was 100% (range 92% to 100 %) ($P < 0.05$). For the same method, specificity was 56% ($P < 0.05$) with a range of 21 to 86 ($P < 0.05$). The positive predictive value (PPV) was 92% (80% to 98%) ($P < 0.05$). The negative predictive value (NPV) for the ELISA-based pregnancy method was 100% (48% to 100%) ($P < 0.05$). The sensitivity of ELISA-based plasma PAGs ($n = 54$) was recorded as 98% (88% to 100%) and specificity was 56%. Positive predictive value was 92% (80-98%) ($P < 0.05$). Negative predictive value was as 83% (36 - 100%) ($P < 0.05$) which was comparable with milk-based PAGs method. The third PD was carried out by measuring the P₄ concentration with RIA. The sensitivity of this method was 98% (88 - 100%) ($P < 0.05$) and sensitivity was 89%. The positive predictive value (PPV) was 98% (88 - 100%) ($P < 0.05$) and NPV was 89% (52 - 100%) ($P < 0.05$).

Table 3 Optical density from the assay of pregnancy associated glycoproteins in milk and plasma and progesterone concentration in postpartum, pregnant dairy cows

Diagnosis	PAGs in milk, OD	PAGs in plasma, OD	P ₄ in plasma, ng/mL
True pregnant	45	44	44
False pregnant	4	4	1
True non-pregnant	5	5	8
False non-pregnant	0	1	1
Assay characteristics ¹			
Sensitivity	100	98	98
Specificity	56	56	89
Positive predictive value	92	92	98
Negative predictive value	100	83	89

¹ Sensitivity: percentage of animals diagnosed as pregnant that were truly pregnant; specificity: percentage of animals diagnosed as not pregnant that were truly not pregnant; positive predictive value: ability to correctly identify pregnant animals, negative predictive value: ability to correctly identify non pregnant animals
PAGs: pregnancy-associated glycoproteins, P₄: progesterone. OD: optical density

Foetal sex has been shown to affect the PAG profile in the peri-partum period (Gabor *et al.*, 2007). However, Serrano *et al.* (2009) and López-Gatius *et al.* (2007) found no such effects from days 40 to 210 of pregnancy. In the present study, no effect was observed of the sex or the sire of the foetus on the profile of milk or plasma PAGs (Figure 1).

The P₄ level in serum was positively correlated with the concentration of PAGs in milk and in plasma ($r = 0.46$ and 0.42 , respectively). The levels of PAGs in milk and plasma were also highly correlated ($r = 0.85$).

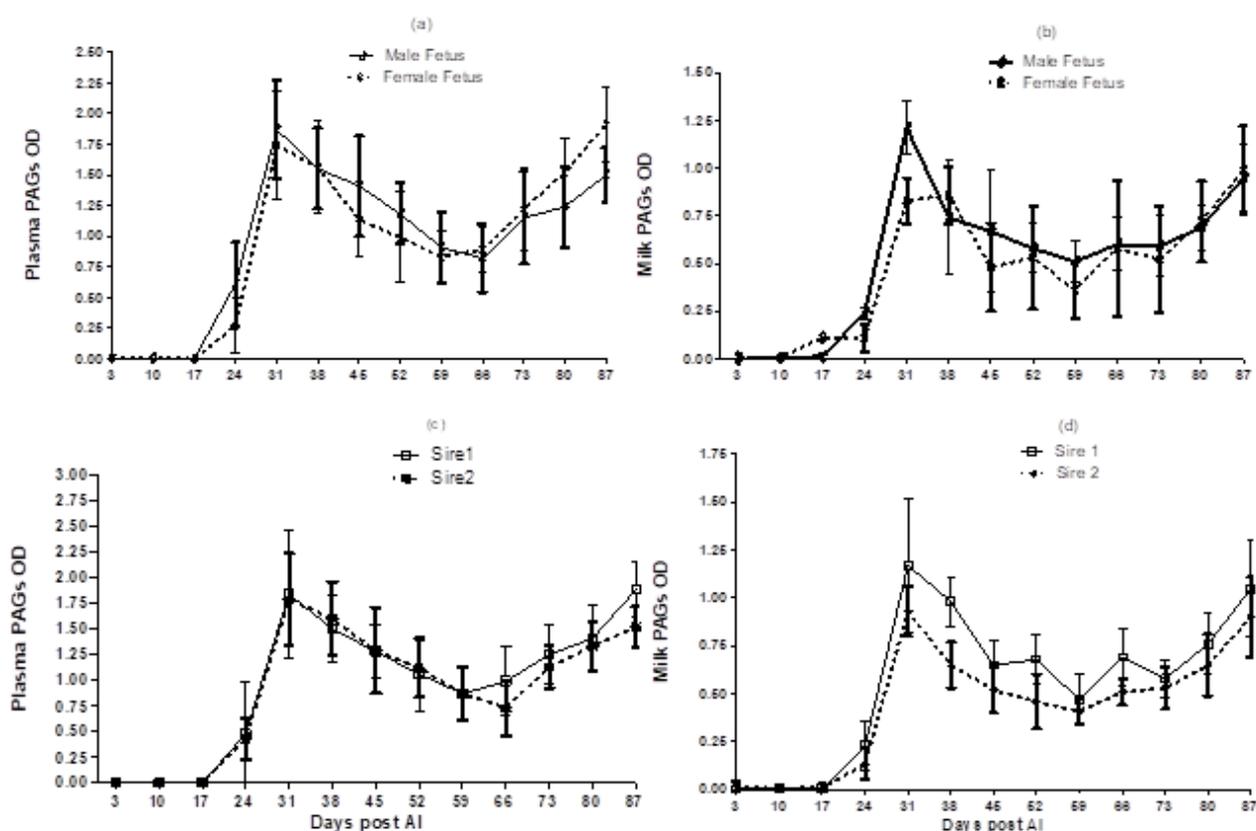


Figure 1 Effect of foetal sex on (a): plasma PAG profile (optical density, OD) and (b): Milk PAGs profile; effect of sires on (c): Plasma PAGs profile and (d): Milk PAGs profile on weekly basis in Holstein postpartum dairy cows during first trimester of gestation period

In Study 2, the difference between synchronization protocols was not significant ($P < 0.05$) for OD of the plasma PAGs on days 30, 45 and 75 post AI, but was significant on days 60 ($P < 0.01$) and 90 ($P = 0.01$) (Table 4). The difference between synchronization protocols in optical density of PAGs measured in milk was not significant except on day 75 ($P = 0.02$) post AI. The difference in synchronization protocols did not affect P_4 levels at any point.

Table 4 Pregnancy associated glycoproteins in plasma and milk and the plasma concentration of progesterone on days 30, 45, 60, 75 and 90 post AI in lactating dairy cows synchronized by G7G-Ovsynch and Ovsynch protocols in Study 2

Days post AI	G7G-Ovsynch			Ovsynch		
	PAGs in plasma	PAGs in milk	P_4	PAGs in plasma	PAGs in Milk	P_4
30	0.85 ± 0.12	1.43 ± 0.12	5.84 ± 0.23	0.75 ± 0.08	1.54 ± 0.12	5.74 ± 0.35
45	0.56 ± 0.08	1.35 ± 0.06	6.47 ± 0.20	0.45 ± 0.05	1.23 ± 0.12	6.66 ± 0.32
60	0.59 ± 0.06	1.18 ± 0.09	7.90 ± 0.22	0.35 ± 0.04	1.10 ± 0.12	7.73 ± 0.22
75	0.62 ± 0.15	1.36 ± 0.09	7.74 ± 0.30	0.50 ± 0.04	1.07 ± 0.06	7.60 ± 0.41
90	0.86 ± 0.08	1.66 ± 0.08	8.47 ± 0.27	0.61 ± 0.04	1.74 ± 0.08	8.29 ± 0.30

PAGs: pregnancy associated glycoproteins, P_4 : progesterone

The results of the present studies are in accord with the findings of Dirandeh *et al.* (2015), who used an initial injection of prostaglandin or GnRH to induce ovulation, followed by GnRH six or seven days later, with the use of prostaglandin being more effective. The recorded PR was approximately 41% on day 32 post AI and approximately 35% on day 60. Thus, 6% of the pregnancies were lost between day 32 and day 60. Based on these results, Dirandeh *et al.* (2015) concluded that G7G-Ovsynch, which was practical in execution, could be used with confidence as most injections were given on the same day of the week. In the present study, the rates of pregnancy and pregnancy loss were slightly greater at comparable times than in Dirandeh *et al.* (2015).

A non-invasive tool to detect pregnancy early in gestation is an important factor in optimal reproductive management. During the last three decades PAGs have proved a suitable indicator of pregnancy for use early in gestation (Sasser *et al.*, 1986). Besides being an indicator of pregnancy, the PAG profile is useful in assessing placental competency and the successful continuation of pregnancy. An atypical PAG profile may indicate embryonic mortality and loss of a pregnancy (Butler *et al.*, 1982; Friedrich & Holtz 2010; López-Gatius *et al.*, 2007; Pohler *et al.*, 2013). Pohler *et al.* (2013) documented overall 19% LEM. Approximately 50% of late embryonic mortality occurs between days 28 and 42 post AI (Silke *et al.*, 2002) and results in a prolonged post-partum interval to rebreeding. From a managerial point of view, accurate pregnancy diagnosis is essential in minimizing late embryonic mortality (Pohler *et al.*, 2013). These authors observed that on day 31 post AI, PAGs were a reasonable indicator of pregnancy and might provide insight into the successful maintenance of pregnancy beyond this point.

There was a positive correlation between P₄ profile and milk or plasma PAGs in the present study. Pregnancy-associated glycoproteins appear to regulate luteal secretion of P₄ from days 50 to 90 of pregnancy through the stimulation of prostaglandin E secretion (Weems *et al.*, 2007). In the present study, PAGs were detectable in the third week of gestation, increased through the fourth week, and then decreased. After this declining phase, there was a gradual rise in PAGs until the last day of sampling in the present study.

High sensitivity is an essential feature of any EPD test. In the present study, the percentage of animals diagnosed as pregnant that were truly pregnant was 100% and 98% in tests that used milk and plasma. Thus, these tests were highly sensitive. However, the percentage was higher of animals diagnosed as not pregnant that were truly not pregnant. Therefore, the specificity of these pregnancy tests was lower. Truly pregnant animals that were diagnosed as not being pregnant might be subjected to another round of hormonal treatment, which could result in the loss of their pregnancy and a prolonged intercalving period. In the same way, low specificity leads to misdiagnosis of cows as pregnant when they should be in the true non-pregnant category according to their real pregnancy status, which also lengthens their intercalving period. Like the present results, Giordano *et al.* (2013) found the sensitivity of EPD with PAGs 1.8 times greater than the specificity of the test. Any test that has an error rate that is equal to or less than 3% is deemed acceptable as a tool for PD. Ferguson and Galligan (2011) considered that any test that had less than 90% sensitivity was not viable. Giordano *et al.* (2013) proposed stricter criteria with the suggestion that sensitivity be greater than 96% on day 31 ± 3 and greater than 94% on day 24 ± 3 post AI.

Conclusion

Pregnancy-associated glycoproteins in milk and plasma were at least as accurate as ultrasonography in identifying pregnant and non-pregnant Holstein cows at the end of the first month of gestation. Thus, PAG-based tests could serve as a viable alternative for EPD by dairy farmers who are unable to obtain veterinary assistance. The G7G-Ovsynch protocol should be evaluated in animals with more than three parities. Given the small number of observations, no definitive conclusion could be drawn from the observed 6% difference between synchronization protocols in the PR of cows in their first lactation. However, the observed numerical advantage in PR with the G7G-Ovsynch protocol may make it the protocol of choice in postpartum cows.

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Author Contributions

AHS and DLR executed the project under the supervision of WJS in study 1. YN and DN executed, analysed and interpreted the data in study 2. AHS collected blood samples and compiled the data. AS supervised compilation of data. WJS and YN conceived and planned experiments. IA, RSB, NA, AHS, MSY and SA analysed and interpreted the data, wrote the manuscript and prepared the figures.

Conflict of Interest Declaration

The authors have no conflict of interest to declare.

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