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SHORT COMMUNICATION

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Bovine milk sampling efficiency for pregnancy-associated glycoproteins (PAG) detection test

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Abstract

Two experiments were conducted to verify whether the time of day at which a milk sample is collected and the possible carryover in the milking system may affect pregnancy-associated glycoproteins (PAG) levels and, consequently, the pregnancy test results in dairy cows. In experiment one, we evaluated the effect of time of day at which the milk sample is collected from 51 cows. In experiment two, which evaluated the possible occurrence of carryover in the milk meter milking system, milk samples from 94 cows belonging to two different farms were used. The samples were subjected to pregnancy test using ELISA methodology to measure PAG concentrations and to classify the samples as positive (pregnant), negative (nonpregnant), or suspicious (recheck). We found that the time of milking did not affect the PAG levels. As to the occurrence of carryover in the milk meter, the PAG levels of the samples collected from Farm-2 were heavily influenced by a carryover effect compared with the samples from Farm-1. Thus, milk samples submitted to a pregnancy test can be collected during the morning or the evening milking. When the sample is collected from the milk meters, periodic equipment maintenance should be noted, including whether the milk meter is totally drained between different animals' milking and equipment cleaning between milking is performed correctly to minimize the occurrence of carryover, thereby avoiding the effect on PAG levels and, consequently, the pregnancy test results. Therefore, a single milk sample can be used for both milk quality tests and pregnancy test.

Additional key words: pregnancy test; milk samples; morning milking; evening milking; carryover; milk meter.

Abbreviations used: ELISA (enzyme-linked immunosorbent assay); ESALQ (College of Agriculture 'Luiz de Queiroz'); κ (kappa); OD (optical density); PAG (pregnancy-associated glycoproteins); USP (University of São Paulo).

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Introduction

The early and accurate detection of the reproductive status is an indispensable tool in optimizing the productive and reproductive processes essentials in dairy herds, as the profitability of this activity mainly influences milk yield and, consequently, the reproductive rates of these animals (Whitlock & Maxwell, 2008; Green *et al*., 2009; Lawson *et al*., 2014). The commonly used techniques for detecting the reproductive status of cows involve rectal palpation and transrectal ultrasonography (Silva *et al*., 2007; Whitlock & Maxwell, 2008; Lawson *et al*., 2014). The application of these techniques requires trained and qualified professionals to ensure proper execution (Han *et al*., 2012).

Laboratory tests capable of detecting substances produced during pregnancy are becoming more widespread, for example, the pregnancy test in milk samples using the enzyme-linked immunosorbent assay (ELISA) method to detect and measure pregnancy-associated

glycoproteins (PAG). These PAG are synthesized during pregnancy from the embryo implantation, which enables its detection in the blood or milk, which can be used as a means of diagnosing pregnancy in cows from the 28th day after conception (Green *et al*., 2005; Gajewski *et al*., 2008; Friedrich & Holtz, 2010; LeBlanc, 2013; Lawson *et al*., 2014). As PAG can still be detected in maternal blood circulation in the early post-partum period (residual PAG from a previous pregnancy), the recommendation is that samples collected for these tests must be obtained from animals with more than 60 days in milk to minimize residual PAG detection (LeBlanc, 2013).

A pregnancy diagnosis performed using milk samples exhibits high sensitivity, specificity, and accuracy in detecting the reproductive status (Gajewski *et al*., 2008; LeBlanc, 2013; Lawson *et al*., 2014). Its realization requires a milk sample that can be obtained during the normal milking routine without causing the animal stress or injuries, as well as greater time spent handling the animals, which makes this technique advantageous compared with other diagnostic techniques, as it is a good alternative for dairy farms, especially those that have limited technical support (Han *et al*., 2012).

However, the representativeness of the milk sample subjected to laboratory analysis can be influenced by the way it is collected, especially with regard to the time of day it is obtained and the possible carryover in the milking systems before the collection of the sample (Løvendahl & Bjerring, 2006; Quist *et al*., 2008; Pavel & Gavan, 2011). Milk samples collected during morning milking have a lower fat content compared with samples collected in the evening milking (Reis *et al*., 2007; Quist *et al*., 2008; Forsback *et al*., 2010; Pavel & Gavan, 2011). Differences in the protein levels, which do not change significantly depending on time of milking, were obtained (Quist *et al*., 2008; Løvendahl & Chagunda, 2011; Pavel & Gavan, 2011). As to specific PAG levels, no studies have assessed the effect of the time of day that milking is carried on these levels.

Carryover is capable of causing dilution or contamination of the samples with the residual milk from another animal that has been milked before and has remained stored in the milk meter or in the milking equipment tubing (Løvendahl & Bjerring, 2006). This effect can be avoided by carrying out regular maintenance such that all milk is drained from the meter between different animals' milking, combined with good practices of cleaning the equipment between milking (Løvendahl & Bjerring, 2006). Both the milking time and the occurrence of carryover in the milk meter can have a great impact on the laboratory test results and lead to a bias in the representative results of the herds in question.

As the ELISA test in milk samples is a commercially new methodology, awareness of how different collection strategies can influence PAG levels, and consequently the pregnancy test results, are unknown. Thus, this study aimed to verify whether the time of day that the sample is collected (morning milking or evening milking) and the possible occurrence of carryover in the milk meter milking system have an impact on PAG levels and, consequently, on the pregnancy test results.

Material and methods

First experiment

This experiment aimed to determine whether the milking time of sample collection (in the morning or evening milking) affects the PAG levels and consequently the pregnancy test results. Milk samples (40 mL, n = 102) were collected from 51 cows (two samples per animal, one obtained from the morning milking and one from the evening milking), belonging to the dairy herd of the Animal Science Department, College of Agriculture 'Luiz de Queiroz' (ESALQ), University of São Paulo (USP). The herd was composed of Holstein and crossbred cows housed in a tie stall feedlot (n = 12) or pasture (n = 39). A comparison of the results of the pregnancy test in milk samples with other diagnostic methods was not included in the objectives of this study and therefore the reproductive status and the days of pregnancy during the sampling period were unknown. The milk samples were collected directly from the milk meter milking equipment (DeLaval® mechanical milking) immediately at the end of each animal milking in sterile plastic bottles of 50-mL capacity. In each sample bottle, one bronopol tablet (2-bromo-2-nitropropane-1,3-diol) was added as a preservative. The samples were analyzed on the same day they were collected in the laboratory of Clínica do Leite, ESALQ/USP. The relative PAG levels were determined using a commercial ELISA test (Milk Pregnancy Test, Idexx®) available in kit form, which qualitatively ranks the samples as negative (nonpregnant), positive (pregnant), and suspicious (requires a new verification) based upon the predetermined optical density (OD) limits (negative OD < 0.100; suspicious 0.100 ≤ OD < 0.250 and positive OD ≥ 0.250). The optical density readings correspond to the relative PAG level in the sample. The data were organized in a double-entry table, where each entry was one of the assessments. The agreement between the pregnancy test result in the milk samples collected in the morning and the evening was determined by calculating the

kappa (κ) using R software (R Core Team, 2015). A kappa value between 0.41 and 0.60 indicates moderate agreement between the results, 0.61 to 0.80 indicates substantial agreement, and greater 0.81 indicates almost perfect agreement (Watson & Petrie, 2010).

Second experiment

The objective of this study was to verify the possible occurrence of carryover in the milk meter, and its effect on the PAG levels in milk samples and consequently the pregnancy test results. Milk samples were collected from two different herds. The first set of samples were collected from a commercial farm located in Piracicaba, São Paulo, which used a mechanical milking system GEA® with a "Metatron" milk meter (Farm-1). The herd consisted of Holstein cows confined in a compost barn system. In this farm, 42 cows' milk samples were collected, one directly collected from the teat (after the pre-dipping and before milking) and one collected from the milk meter (immediately at the end of milking). The second herd belonged to the Animal Science Department of ESALQ/USP, which used a mechanical milking system DeLaval® with a "Mark 5 Milk Meter" (Farm-2). The herd was composed of Holstein and crossbred cows in pasture. The samples were collected from 52 cows following the same methodology of the previous farm. The animal milking order was recorded so that the carryover could be analyzed. The samples were stored, preserved and analyzed using the same methodology described in the previous experiment, and the results were analyzed as previously described.

Results and discussion

The first experiment was conducted to evaluate the effect of collection time of the milk sample on PAG levels and, therefore, on the results of the pregnancy test. From the 102 milk samples collected and submitted to the pregnancy test, 61% had a positive result, independent of milking time; 31% were classified as negative in the morning milking sample, whereas 33% were negative in the evening milking sample. Further, 8% of the samples were suspicious (required additional verification) when collected in the morning, whereas 6% were classified this way when collected from the evening milking (Table 1).

This variation in the results of the negative and suspicious samples can be explained by the change in classification of the samples from a unique cow, which in the morning milking sample was classified as suspicious, and in the evening milking as negative. This small change resulted in kappa equal to 0.96, which indicates that the correlation between the pregnancy test results of the samples collected at different times of the day was almost perfect.

The samples of this unique cow that showed varied PAG levels between the samples presented an optical density (OD) of 0.12 in the sample collected in the morning and 0.05 in the sample obtained in the evening (the cutoff point for a sample to pass a negative rating for suspicion is 0.10). This small increase in the PAG content in the milk collected in the morning differs from the results of studies that analyzed the total protein content in milk samples, which found that protein levels remained constant and did not differ depending on the milking time (Fava *et al*., 2011; Pavel & Gavan, 2011). This variation between milking times was observed only for one animal, and thus was unable to derail the test.

The total protein levels did not show a significant variation between milk samples collected in the morning and those collected in the evening milking (Quist *et al*., 2008; Løvendahl & Chagunda, 2011; Pavel & Gavan, 2011). As the PAG are glycoproteins and are included in the total protein levels measured, they also showed no variation in the levels when observed in the samples collected at different times of day.

Experiment two was conducted to check the possible occurrence of carryover in the milking equipment, its effect on PAG levels in the milk samples, and, therefore, the pregnancy test results. When the occurrence of a carryover effect was measured, variation was noted according to the milking equipment and milk meter used (Table 2).

In the analysis carried out with the milk from Farm-1, of the direct samples collected from the teat, 50% were classified as positive and 50% negative, whereas in the samples collected from the milk meter

Table 1. Results of the ELISA test on milk samples collected during the morning and evening milkings.

	Pregnant	Nonpregnant	Recheck	Total
Morning milking	31	16	4	51
Evening milking	31	17	3	51
Kappa*	-	-	-	0.96

*Kappa = test of agreement between periods tested.

Table 2. Results of the ELISA test on milk samples collected at Farm-1 and Farm-2.

	Pregnant	Nonpregnant	Recheck	Total
		Farm-1		
Milk meter	21	18	3	42
Teat	21	21	0	42
Kappa*	-	-	-	0.86
		Farm-2		
Milk meter	37	8	7	52
Teat	36	10	6	52
Kappa*	-	-	-	0.66

*Kappa = test of agreement between the collection form tested.

at the end of milking, the percentage of negative samples decreased to 43% because 7% of them had become suspicious. This variation between the test results on the samples collected directly from the teat and those from the milk meter led to a kappa index of 0.86, or an almost perfect agreement between the pregnancy test results (Table 2).

The variations among the pregnancy test results cannot be justified by the time wherein the data were collected (beginning of milking or from the milk meter) because, as described by Lollivier *et al*. (2002), protein and lactose levels did not show variation during milking, unlike fat levels. Thus, samples collected at the beginning or end of the milking must have similar levels of proteins and, consequently, PAG.

Therefore, the occurrence of these variations was characterized by carryover between these samples. When the results of the optical density of these samples were analyzed, we discovered that the three cows in which the milk presented variation were milked in sequence of pregnant cows with high levels of PAG (OD = 3.76, 3.23, and 1.41). The probable residual milk permanence of these animals on the meter or tubing was possibly responsible for the increased PAG concentrations.

A milk sample was also influenced by a carryover effect in studies conducted by LeBlanc (2013), in which the cow had been diagnosed by transrectal ultrasonography as nonpregnant and was considered positive for the pregnancy test through the measurement of PAG. The OD result of this milk sample was 0.28, whereas the cutoff for the cow to be classified as positive is 0.25. The author found that the occurrence of false-positive sample contamination with residual milk present in the milking equipment's meter occurred because the cow that was previously milked was positive.

When the results of the pregnancy test using milk collected from Farm-2 were analyzed, an even greater influence of carryover was noted. As seen in Table 2, the classification of direct samples collected from the teat before the milking and from the milk meter collected immediately at the end of milking showed substantial agreement.

From milk samples collected directly from the teat at Farm-2, 69% were positive, 19% were negative, and 12% were classified as suspicious, whereas in the samples collected from the milk meter at the end of milking, the percentage of positive samples increased to 71%, with the negative samples decreasing to 15% and suspected samples increasing to 14%. This variation between the pregnancy tests resulted in a kappa lower (k = 0.66) than that found in Farm-1, and the correlation between the two test results decreased to substantial.

In total, eight animals had a different classification between the test result of the direct sample collected from the teat and that collected from the milk meter. The negative cows that changed status to suspect (n = 3) and suspect to positive (n = 3) were collected following the milking of positive cows with high levels of PAG. The residual milk from these cows likely resulted in the increased levels of PAG in the samples with these variations. One sample changed from positive to suspect, and another from positive to negative. In these cases, the cows with lower levels of PAG had previously been milked, and the residual milk of these possibly caused a dilution effect in the subsequent samples, reducing the PAG levels and causing a variation in the results. Unfortunately, the results of a reproductive diagnosis via ultrasound was not informed by none of the farms, making it impossible to confirm these strong assumptions.

The main cause of the carryover is likely due to poor regulations and maintenance of milking equipment and milk meters, combined with incorrect hygiene practices. Importantly, on the days when the samples were taken at Farm-2, the employees reported washing the equipment before the start of milking and at the end of milking with only hot water and without the addition of detergent. This procedure may have been responsi-

ble for the observed variation in the pregnancy test results using milk samples from this farm.

Variations between the results and consequences of erroneous classifications of reproductive status (false positives and false negatives) were also reported by Szenci *et al*. (1998), Silva *et al*. (2007), Green *et al*. (2009) and Friedrich & Holtz (2010). Friedrich & Holtz (2010) found a false-negative result of 7% using the measurement of PAG. For this reason, the reproductive history of the animals that will be sampled for the test must be considered, as well as the post-partum period necessary (minimum 60 days in milk) to avoid the residual PAG of previous pregnancies, which can interfere with the test and increase the rate of erroneous results.

The effect of a sample being misclassified varies depending on the status to which it is assigned. Negative samples classified as suspicious will delay the reintroduction of these cows into breeding programs, increasing the interval between calving (when the sample is classified as suspicious, it is recommended to perform the test again after a few weeks). If the sample is suspected and the test classes it as negative, this animal will be reintroduced into the breeding programs, and the use of hormones will cause embryonic or fetal loss (if the animal really was pregnant and the PAG levels were still increasing). For these reasons, periodic maintenance, ensuring complete draining of the milk meter between milking of different animals and the adequate cleaning of milking equipment, is essential to prevent classification errors caused by contamination or dilution of milk by residual samples from other animals.

In summary, the time at which the milk sample is collected does not affect the levels of PAG. Thus, milk samples submitted to the pregnancy test can be collected both in the morning and in the evening milking. When the sample is collected from the milk meters, maintenance of the equipment should be noted, and drainage and sanitization of the milk meter between milking of different animals must be performed correctly to prevent carryover that can consequently affect the pregnancy test results. The same milk sample obtained for milk quality analysis can also be used for pregnancy testing.

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