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Storage strategies and processing of bovine milk samples for pregnancy-associated glycoproteins detection test

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ABSTRACT

Three experiments were performed to test if the time and storage conditions of milk samples, the preheating of samples in a water bath, as well as the carryover effect in laboratory analysis equipment could affect the pregnancy-associated glycoproteins (PAG) levels, and consequently the results of a pregnancy test. The pregnancy test used in both experiments uses the enzyme-linked immunosorbent assay method to measure the concentrations of PAG and classify the samples as pregnant, nonpregnant or suspicious. As a result, PAG levels showed no variation when the samples were analyzed up to 9 days after collection, whether stored in ambient temperature or refrigerated. The preheating of the samples in a water bath and prior to the analysis of SCC and composition also did not affect the levels of PAG, allowing the same sample used in the quality analysis to be used for the pregnancy test.

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Carryover effect; milk sample; pregnancy test; sample age; storage conditions; water bath

Introduction

The early and accurate detection of reproductive status is an essential tool for the optimization of the productive and reproductive processes in dairy herds (Whitlock & Maxwell, 2008; Green et al., 2009; Lawson et al., 2014). The inability to perform early diagnosis of pregnancy can result in economic losses in milk production systems, mainly due to an increase in the calving interval, a drop in milk production, fewer calves born throughout the reproductive life of the cow and additional spending on unproductive animals (Neves et al., 2008; Palhano, 2008).

The techniques most used for pregnancy diagnosis of dairy cows are 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 correct execution (Han et al., 2012), which means that on farms where technical support is limited, early pregnancy diagnosis is difficult.

Currently, laboratory immunoassays, such as radioimmunoassay and enzyme-linked immunosorbent assay (ELISA), which are capable of detecting and measuring pregnancy-associated glycoproteins (PAG), are increasingly being applied in early pregnancy diagnosis in cattle. The PAG are synthesized during pregnancy starting from embryo implantation, which enables its detection in the blood or milk and can be used as diagnostics from the 28th day post conception (Green et al., 2005; Gajewski et al., 2008; Friedrich & Holtz, 2010; LeBlanc, 2013; Lawson et al., 2014). A pregnancy diagnosis performed using milk samples exhibits high accuracy, sensitivity and specificity in detecting the reproductive status, 99.5%, 99.2% and 100%, respectively (Lawson et al., 2014).

The measurement of these PAG levels as reproductive diagnosis in milk samples brings benefits to the producer, especially in minimizing labor and time spent on handling animals when compared to palpation, ultrasound or blood tests (Han et al., 2012). The milk sample used in the pregnancy test can be obtained during a normal milking routine without causing stress, injuries and increased chance of embryonic or fetal loss by excessive manipulation of the reproductive tract (Han et al., 2012; Lawson et al., 2014).

However, the method of collection, conditions and time of storage, and the laboratory procedure used can influence the representativeness of the milk sample (Meyer, 2003). Samples used for quality analyses should be diluted with preservative and refrigerated immediately after collection (Meyer, 2003). These samples

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suffer from preheating in a water bath before being analyzed by the laboratory equipment. In addition, there is a possibility of the carryover effect, which is capable of causing dilution or contamination of samples with the residual milk of previous samples analyzed (Byrem & Voisinet, 2013).

The pregnancy test of milk samples using the ELISA method is a new commercially available technique. It is not known how different storage strategies and processing of these samples can influence the levels of PAG. Thus, this study aimed to test whether the conditions and time of storage, preheating in a water bath and the possible carryover effect in laboratory analysis equipment has an impact on the levels of PAG, making it impossible to use the same sample for both the quality analyses and the pregnancy test.

Materials and methods

To detect if the previous analysis for SCC and composition could affect PAG levels of the milk samples, three experiments were performed to evaluate the impact of these analyses on the results of the pregnancy tests.

First experiment

The objective of this study was to verify the effects of storage temperature and the age of milk samples on the PAG levels and consequently on the results of the pregnancy test. Milk samples (160 mL, $n = 40$) were collected at a commercial farm located in Piracicaba, São Paulo. The milk was collected directly from the milk meter of the milking equipment (GEA® mechanical milking, Düsseldorf, Germany) immediately at the end of each animal milking in sterile cylindrical plastic bottles of 200 mL (Embalpharma®, Pescaria Brava, Santa Catarina, Brazil). In each sample bottle, four bronopol tablets (Broad Spectrum Microtabs II, 8 mg bronopol and 0.30 mg natamycin, Advanced Instruments®, Norwood, Massachusetts, United States) were added as a preservative (one for each 40 mL of milk), as recommended by the producer of the ELISA kit for pregnancy detection used in this study. The comparison of the results of the pregnancy test in milk samples with other diagnostic methods was not included in the objectives of this study; therefore, the reproductive status and the days of pregnancy during the sampling period were unknown.

At the end of the collection, the samples were sent to the laboratory of Clínica do Leite, ESALQ-USP (Luiz de Queiroz School of Agriculture, University of São Paulo, Piracicaba, São Paulo, Brazil). Each sample was divided into seven subsamples, one was analyzed on the day of collection (day 0 – control sample), three were stored at an average temperature of 26°C (ambient temperature) and three were stored at average temperature of 7°C (refrigerated).

The PAG levels were determined on days 0, 3, 6 and 9 (D0, D3, D6 and D9) after collection, at both storage temperatures using a commercial ELISA test (Milk Pregnancy Test, Idexx®, Westbrook, Maine, United States) available in kit form, which qualitatively ranks the samples as negative (nonpregnant), positive (pregnant) and suspicious (recheck – requires a new verification), based upon the predetermined optical density (OD) limits (negative $OD < 0.100$; suspicious $0.100 \leq OD < 0.250$ and positive $OD \geq 0.250$). The OD readings correspond to the relative PAG levels 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 and the samples analyzed with different ages and storage temperatures was determined by calculating the Kappa index (κ) using R software (R Core Team 2015). A kappa value between 0.41 and 0.60 indicates moderate agreement between the results, 0.61–0.80 indicates substantial agreement and greater 0.81 indicates almost perfect agreement (Watson & Petrie, 2010).

Second experiment

This experiment aimed to verify the influence of preheating milk samples in the water bath on the results of pregnancy tests. Milk samples (40 mL, $n = 14$) were collected from the dairy herd of the Animal Science Department, ESALQ/USP. The samples were collected using the milk meter milking equipment (DeLaval® mechanical milking, Tumba, Sweden) in sterile cylindrical plastic bottles of 50 mL (Embalpharma®, Pescaria Brava, Santa Catarina, Brazil) immediately after milking each animal. In each sample bottle, one bronopol tablet (2-bromo-2-nitropropane-1, 3-diol) was added as a preservative. The reproductive status and the days of pregnancy during the sampling period were unknown.

The samples were sent to the laboratory of Clínica do Leite, ESALQ-USP. Each sample was divided into two subsamples. Of these, one remained in the water bath for 30 min at 40°C (±2°C) and the other was maintained under refrigeration (control sample). The relative levels of PAG were determined in all samples using the same methodology described in the previous experiment. The processing and analysis of the pregnancy test results were also the same as described in the previous experiment.

Third experiment

This experiment aimed to test the possible occurrence of the carryover between samples in the laboratory analysis of milk quality equipment, its effect on the PAG levels and consequently on the pregnancy test results. Milk samples were collected from 10 nonpregnant cows and one pregnant cow, belonging to the dairy herd of the ESALQ/USP. The reproductive status of females was passed on by the farmer, who examined it using transrectal ultrasonography (DP-2200 Vet, Mindray®, Shenzhen, China) as a pregnancy diagnosis.

The samples of milk from the nonpregnant cows (80 mL, $n = 10$) were collected using the milk meter of the milking equipment (DeLaval® mechanical milking), immediately at the end of each animal milking, in plastic bottles into which previously two bronopol tablets (2-bromo-2-nitropropane-1, 3-diol) had been added as a preservative. One sample of 1 L milk was obtained from the pregnant cow. This cow was milked separately, having her milk directed to an individual milk jar, which allowed homogenization before collection. In this sample bottle, 25 preservative tablets were added.

The samples were sent to the laboratory of 'Clínica do Leite', ESALQ-USP, to perform the analysis. Each 80 mL sample of the nonpregnant cows was divided into three subsamples plus a control sample. The sample of 1 L of the pregnant cow was divided into 30 subsamples plus a control sample.

Each animal had a milk sample analyzed by three equipment performing milk quality analysis Combifoss – EQ1 (79,910, Foss®, Hillerød, Denmark), Combidelta – EQ2 (Soma Scope, Delta®, Drachten, The Netherlands) and Combibentley – EQ3 (CC-300, Bentley®, Chaska, Minnesota). In total, 20 samples were analyzed in each equipment, 10 with the milk of the pregnant cow and 10 with the milk of the nonpregnant cows. The order followed for the analyses was as follows: (1) milk sample of the pregnant cow; (2) collect the milk sample the first nonpregnant cow; (3) milk sample of the pregnant cow; (4) collect the milk sample the second nonpregnant cow, and so on. The control samples were not analyzed by this equipment.

All the samples (those that have passed through the equipment and the control samples), were processed, analyzed and their PAG levels were determined using the same methodology described in previous experiments.

Results and discussion

Influence of time and storage conditions on milk samples

The conditions and time of milk sample storage until analysis may affect its composition and thus the representativeness of the results obtained in these analyses (Meyer, 2003). The protein content suffers less influence of temperature and storage time when compared to the levels of other milk constituents (Meyer, 2003; Cassoli et al., 2010). Cassoli et al. (2010), found no variation in protein levels when samples were analyzed up to seven days after collection, as long they were preserved with bronopol, maintained under refrigeration or at ambient temperature.

In this study, milk age (0, 3, 6 and 9 days) and the tested storage temperatures (ambient and refrigerated) had no impact on the levels of PAG. As shown in Table 1, the classification of the samples analyzed using a pregnancy test from day 0 to 9 at the ambient temperature. The samples analyzed exhibited a high degree of concordance among days. The milk status results did not differ from ages and temperatures analyzed, so we decided to present just the results from ambient temperature, because it is an extreme situation compared to refrigerated.

The results showed that 55% of the samples were positive and 45% negative, regardless of the age and the condition in which they were stored. These identical observations between the state of the milk at different ages and storage temperatures enabled a Kappa number equal to 1, which indicates a high correlation of the results of the pregnancy test.

The PAG levels remained constant when the samples were preserved with bronopol, analyzed for up to 9 days after collection and stored at the ambient temperature or refrigerated. This study shows that the total protein levels in milk as well as the PAG levels did not vary to the same extent as fat and other milk constituents did (Meyer, 2003; Cassoli et al., 2010). These results were similar to those found by Cassoli et al. (2010), who analyzed and detected no variation in the protein content of milk samples until the 7th day after collection, and also to Meyer (2003), who evaluated the protein levels of stored milk samples that were refrigerated, and these did not change until the 15th day after collection.

Preheating influence

When laboratory analyses on milk quality are carried out, the samples are subjected to preheating of

Table 1. Results of the ELISA test on milk samples stored from day 0 to 9 at ambient temperature.

Day	Positive	Negative	Suspicious	Total
0	22	18	0	40
3	22	18	0	40
6	22	18	0	40
9	22	18	0	40
Kappa[a]	–	–	–	1

[a]Kappa = test of agreement between days tested.

Table 2. Results of the ELISA test on preheated milk samples in a water bath compared to the control samples.

	Positive	Negative	Suspicious	Total
Control	7	6	1	14
Preheated	7	6	1	14
Kappa[a]	–	–	–	1

[a]Kappa = test of agreement between situation tested.

approximately 30 min at 40°C in a water bath. To verify if the preheating of milk samples causes an effect on PAG levels, the results of the pregnancy test samples that were heated in a water bath were compared to their respective controls results (Table 2).

The results showed that 50% of the samples were positive, 43% were negative and 7% were classified as suspicious, regardless of whether they had been preheated. These identical observations between samples that underwent preheating and control samples enabled the Kappa index to equal 1, which indicates a high correlation in the results of the pregnancy test.

It is important to determine if the samples used for quality analysis may be or not used subsequently for the pregnancy test. According to the results of this experiment, the levels of PAG were not affected by preheating in a water bath and thus, the sample that was previously analyzed for quality (SCC and composition) can also be used for the pregnancy test.

Influence of carryover on laboratory analysis equipment

The ELISA tests were carried out to identify the carryover effect in laboratory equipment. These results can be seen in Table 3, which shows the correlation between the results of the control samples with those that were analyzed by equipment 1 and then subjected to the pregnancy test. As the results of samples submitted to the three equipment did not differ, we chose to present the data from only one equipment (EQ1).

The results showed that 50% of the samples were classified as positive, 45% negative and 5% as suspicious in both control samples and in samples previously analyzed by the laboratory equipment. These same results made the kappa equal to 1, which means that the correlation between the results of the pregnancy test performed in the control samples and the previously analyzed samples for quality was high. Thus, the prior analysis of the milk samples did not influence the outcome of the pregnancy test and the occurrence of the carryover in the analyzed samples was not observed.

Table 3. Results of the ELISA test performed on the milk samples processed by equipment 1 before a pregnancy test compared to the control samples.

	Positive	Negative	Suspicious	Total
Control	10	9	1	20
Samples pre-analyzed by equipment 1	10	9	1	20
Kappa[a]	–	–	–	1

[a]Kappa = test of agreement between situation tested.

One of the 10 nonpregnant cows (status pre-defined by veterinary examination) used in this study showed a different status as told by the farm. When this sample was analyzed using the pregnancy test through the measurement of PAG levels, the result was suspicious in both the control sample and the sample pre-analyzed for quality and composition.

Conclusions

The samples submitted to the laboratory quality analysis can also be used for the pregnancy test. The PAG levels were not affected by preheating in a water bath or by carryover in laboratory analysis equipment. Milk samples preserved with bronopol and maintained refrigerated or at ambient temperature can be analyzed up to 9 days after collection.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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