

**Sensitivity and specificity of three pregnancy-associated glycoprotein ELISA assays and factors that affect test accuracy in South African dairy cows**

*By*

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## E. Abbreviations

|       |  |
|-------|--|
| °C    | : Degrees Celsius                          |
| %     | : Percentage                               |
| AI    | : Artificial Insemination                  |
| BPAG  | : Bovine Pregnancy-Associated Glycoprotein |
| DIM   | : Days in milk                             |
| ELISA | : Enzyme-Linked Immunosorbent Assay        |
| HRPO  | : Horseradish Peroxidase                   |
| MHz   | : Megahertz                                |
| OD    | : Optical density                          |
| PSPB  | : Pregnancy-Specific Protein-B             |
| S-N   | : Sample minus Negative                    |
| TMB   | : 3, 3', 5, 5'-Tetramethylbenzidine        |
| TRUS  | : Transrectal Ultrasonography              |



## F. Summary

The sensitivity and specificity of three pregnancy-associated glycoprotein ELISAs and factors affecting tests' accuracy in South African dairy herds

By

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The ruminant trophoblast produces pregnancy-associated glycoproteins (BPAGs) starting at day 25 after conception and continuing until parturition. These glycoproteins are detectable in the blood and milk of pregnant females and can be used to diagnose and monitor pregnancy in cattle, small stock and buffaloes. The objective of the study was to estimate the accuracy of three enzyme-linked immune-sorbent assays (ELISA) for BPAGs to diagnose pregnancy in dairy cattle under South African field conditions compared to transrectal ultrasound (TRUS) as a gold standard. A multi-centre prospective study was conducted at five sites in South Africa utilizing the assistance of experienced veterinary practitioners. A total of 1036 plasma and milk samples from dairy cows were analysed. Transrectal ultrasonography was performed immediately after sample collection. The milk and blood samples were analysed with commercial ELISA kits. A total of 532 and 395 cows were found to be pregnant and open, respectively based on ultrasound. The pregnancy rate was 51.4% (532/1036) based on TRUS. Results for sensitivity of ELISA analyses of serum, milk and serum visual ELISA was 99.8%, 99.4%, and 99.6%, respectively. Specificity for the serum, milk and serum visual ELISA was 57.3%, 55.3% and 55.0%, respectively. The results for factors affecting sensitivity and specificity of all the BPAG ELISA analyses (ELISAs) showed that days after AI, breed, DIM and lactation number did not have an effect on sensitivity of any of the BPAG ELISAs. Days after AI and breed influenced the specificity of all the BPAG ELISAs. There was a negative correlation between days after AI and specificity of all the BPAG ELISAs. Specificity decreased gradually as days after AI increased from day 28 to 35. Additionally, pregnancy loss influenced the specificity of the three BPAG ELISAs from 28 to 35 days after AI.

In conclusion, the BPAG ELISAs were sensitive tests for detecting pregnancy relative to TRUS B from days 28-35 and 42-49 d after breeding but poorly specific in identifying non-pregnant cows. The specificity estimates were under-estimated due to the incidence of pregnancy loss during early gestation.

## CHAPTER ONE: LITERATURE REVIEW

### 1.1 Background

Reproductive performance is a critical factor to the viability of a dairy farm. Determining the pregnancy status after breeding is a key element of reproductive management. It is important to confirm pregnancy with a good degree of accuracy. Most importantly, non-pregnant cows should be identified as soon as possible to allow a chance for re-breeding therefore shortening the calving intervals.

There are several methods used to diagnose pregnancy in dairy cows, the two main categories being - physical and chemical methods. The physical methods include rectal palpation (Garmo et al. 2008) and transrectal ultrasonography (Fricke 2002), both which are commonly used. Chemical methods include progesterone (Garmo et al. 2008), oestrone sulphate (Hung, Prakash 1990), pregnancy specific protein B (PSPB) (Han et al. 2012, Romano, Larson 2010) and pregnancy-associated glycoprotein (PAG) (Commun et al. 2016, Breed et al. 2009, Friedrich, Holtz 2010). Other methods besides PAG and ultrasonography is not be discussed further as they are beyond the scope of this dissertation.

This study describes the use of bovine PAG for early pregnancy diagnosis in dairy cows. Bovine pregnancy-associated glycoproteins (BPAG) are produced by the binucleate cells of the bovine embryonic trophoblast (Wooding, Roberts & Green 2005) as early as 21 days after conception and throughout pregnancy. They belong to the aspartic proteinase family (Whitlock, Maxwell 2008). The gene family of bovine BPAGs comprises of 22 transcribed genes and as well as other variants and are expressed at different stages of gestation (Wallace et al. 2015).

Additionally, they are detectable from 25 days of pregnancy until parturition (Green et al. 2005) for diagnosing pregnancy using enzyme-linked immunosorbent assays.

Enzyme-linked immunosorbent assays have been developed to detect BPAGs in blood and milk for diagnosing pregnancy. However, these assays have not been validated in field conditions in the South African dairy industry, nor are veterinary practitioners, farmers and laboratories familiar with their use. Therefore, a validation of these assays is required and recommendations for their use by veterinary practitioners are required.

1.1.1 Application of pregnancy-associated glycoproteins for pregnancy diagnosis in dairy cows  
Pregnancy specific protein B (PSPB) is a placental antigen that was detected in blood of pregnant cows from four weeks after breeding (Butler et al. 1982, Sasser et al. 1986). Other researchers have developed immunoassays that detected similar antigens (Mialon et al. 1993, Zoli et al. 1992). In one case, the antigen was classified as pregnancy associated glycoprotein (PAG) (Zoli et al. 1992). In the other case, the antigen was classified as pregnancy serum 60 (PSP60) because of its molecular weight (Zoli et al. 1991). Due to similarities of PSPB, PSP60 and PAG with the same amino-terminal sequence, it was later concluded to classify the antigen as bovine PAG-1 (Xie et al. 1991) as cited by (Green et al. 2005).

Given the history, BPAGs in blood and milk have since been used as markers for diagnosing pregnancy in cattle. The BPAG levels are believed to rise from 15 to 35 days of gestation and the best time for doing pregnancy diagnosis is between 26 to 30 days of gestation (Zoli et al. 1992, Humblot 2001). In a study by Silva et al. 2007, it was found that the sensitivity and specificity of blood BPAG ELISA ranged from 94-96% and 92-97%, respectively, relative to transrectal ultrasonography at 27 days of gestation. These findings are similar to others (Romano, Larson 2010, Piechotta et al. 2011) where sensitivity ranged from 94-97% and 94-96% and specificity from 97.8% and 91.2% when pregnancy-specific protein B (PSPB) ELISA and BPAG ELISA were used from 28-35 and 26-58 days after AI, respectively. The PSPB and BPAG ELISA performed similarly irrespective of the antigen detected and days after AI.

Other researchers found various responses using the milk BPAG ELISA though sampling was conducted at different gestation days after AI. (Lawson et al. 2014) found that the sensitivity and specificity of the milk BPAG ELISA ranged from 98-100% and 98-100%, respectively, from 30-95 days of gestation. This is similar to the findings of (Commun et al. 2016, Ricci et al. 2015) where the sensitivity ranged from 98.1-100% and 98% and specificity ranged from 90.3-92.3% and 83%, when milk was collected from 30-41 and 32 days of gestation.

#### 1.1.2 Factors influencing pregnancy-associated glycoprotein levels

Several studies have documented on factors associated with BPAG levels in the blood and milk in early pregnancy of dairy cows and how these factors affect the accuracy of pregnancy diagnosis. Possible factors include cow parity, pregnancy loss, milk production (Ricci et al. 2015), lactation number, plasma progesterone, breed of sire, outcome of gestation, season of gestation, day of gestation, foetal number (Serrano et al. 2009), number of AI services (Mercadante et al. 2016).

López-Gatius and co-workers 2007a found that as gestation progresses, cows that carried twins promoted high production of BPAGs in the blood using BPAG A radio-immuno assay (RIA) when sampling was done at 35, 42, 49, 56 and 63 days after AI. This is similar to the findings of Serrano et al. (2009) where plasma BPAG A levels were high in cows carrying twins.

Other researchers found various responses of several factors affecting blood and milk BPAG A levels of dairy cows. In recent studies (Ricci et al. 2015, Mercadante et al. 2016), it was found that plasma BPAG A levels increased with primi-parous cows when compared to multiparous cows and when gestation increased from 32 to 102 day of gestation. On the other hand, the plasma and milk BPAG A levels were decreased after an incidence of pregnancy loss of 13 % for cows diagnosed with singletons (Ricci et al. 2015). This negative influence is also noticed in a study by Mercadante et al. (2016) where pregnant cows that were serviced once had reduced serum BPAG A levels at 32 days after AI compared to pregnant cows that were serviced twice. Nonetheless, it is a challenge to compare different factors in relation to how they influence blood and milk BPAG A levels of dairy cows.

### 1.1.3 Factors influencing pregnancy loss in dairy cows

The fertility of dairy cows is affected by many factors, however, reports have documented that late embryo mortality is the main factor contributing to reproductive inefficiency (Pohler et al. 2016). According to the Committee on Bovine Reproductive Nomenclature (1972), gestation is classified into two periods, the embryonic period which extends from conception to the end of differentiation stage (0-42 days) and the foetal period (42 days until calving). It is reported that one-third of embryos fail to survive during the first 18 days of pregnancy (Zobel et al. 2011). This is because some pregnancies fail due lack of implantation of the embryo to the endometrium. Embryo loss during early pregnancy can be caused by factors such as genetics, physiology and or environmental factors such as nutrition, energy balance and hormones (Shorten et al. 2010).

There are infectious and non-infectious factors that contribute to pregnancy loss in dairy cows. Infectious factors are known to contribute to the incidence of early and late pregnancy loss; however, there is increasing evidence of the role of non-infectious factors in dairy cattle research. A study by López-Gatius et al. 2002 found that twin pregnancy was a risk factor of pregnancy loss from 38 to 90 days of gestation based on ultrasonography and rectal palpation. In the same study, an incidence of pregnancy loss of 21.4% (24/112) was seen in cows that carried twins (López-Gatius et al. 2002). Similarly, others (Silva-del-Río, Colloton & Fricke 2009) found that 11.2% of cows diagnosed with twin pregnancies experienced a single embryo reduction, whereas 13.3% lost both embryos. The major cause of pregnancy loss in cattle was considered to be insufficient uterine space for two embryos (Vanroose et al., 2000 as cited by López-Gatius et al. 2002).

In a study by Lee and co-worker (2007) , it was reported that cows in their third or higher parity suffered a high incidence of pregnancy loss from 28 to 60 days of gestation, which was similar to the findings of Thurmond, Picanso & Jameson (1990) where more pregnancies were lost at fourth calving. Additionally, Humblot (2001) found that cows that were in the 1<sup>st</sup> to 3<sup>rd</sup> parity contributed to pregnancy loss. However, it is unclear how parity influences pregnancy loss in dairy cows. One possibility is that body condition after parturition could be linked to pregnancy loss for high milk producing cows at 3<sup>rd</sup> parity (Lee, Kim 2006). Studies (López-Gatius et al. 2002, Silke et al. 2002) showed that a drop in body condition after parturition increased the risk of pregnancy loss between 28 and 56 days of gestation.

In a recent study by Mercadante et al. (2016), found that low milk yield is associated with pregnancy loss at 32 days of gestation. In the same study it was found that metritis, mastitis and left-displaced abomasum also increased the probability of pregnancy loss for both cows bred during the first and second AI service at 32 days of gestation (Mercadante et al. 2016).

### 1.1.4 Conclusion

This review of literature in this Chapter has concentrated on new technologies that are currently used around the world to check for pregnancy diagnosis in dairy farming and factors that affect

the accuracy of the tests. It has also outlined the prevalence, timing and factors contributing to pregnancy loss, which is commonplace during the same period when pregnancy diagnosis is generally practiced. The BPAG A and PSPB ELISA were reported of high sensitivity and specificity relative to transrectal ultrasonography. To apply this new technology, accuracy should be taken into consideration. Factors such as cow parity, foetal number, clinical diseases, and breed of sire, inseminating bull, season, and milk production have positively and negatively affected levels of pregnancy-associated glycoproteins in both blood and milk. Furthermore, factors such as cow parity, twinning, and reduction in body condition from previous parturition, inseminating bull and multiple diseases were high risk factors associated with pregnancy loss from as early as 28 days of gestation. Thus this chapter provides a basis for investigation of the assays and factors affecting their application in South African dairy cows.

## 1.2 Hypothesis

The use of IDEXX serum, milk, and serum visual BPAG A ELISA are comparable to transrectal ultrasonography for early pregnancy diagnosis in South African dairy cows.

## 1.3 Benefits arising from the project

This project will generate information on the application of pregnancy-associated glycoproteins ELISAs for early pregnancy diagnosis in the South African dairy industry. Additionally, information on factors influencing the accuracy of the assays will be generated. This information will assist dairy farmers and veterinarians by incorporating a diagnostic tool to their planned reproductive programs. Furthermore, this project will generate scientific reports in the form of presentations and publications.

## 1.4 Objectives

- I. To estimate the sensitivity and specificity of the use of serum, milk, and serum visual BPAG ELISAs compared to transrectal ultrasonography;
- II. To determine factors influencing the sensitivity and specificity of the serum, milk, and serum visual BPAG ELISAs for pregnancy diagnosis;
- III. To estimate the incidence of pregnancy loss and determine how this affected the BPAG assay performance.

## CHAPTER TWO: MATERIALS AND METHODS

### 2.1 Study design

A multicentre prospective cohort study was conducted in five important dairy regions of South Africa. These regions were Kwa-Zulu Natal, Mpumalanga, west coast region of Western Cape, western region of Eastern Cape and Eastern Cape. One veterinary practitioner in each study location was recruited.

Each of the five veterinary practitioners was requested to identify one or more large commercial dairy herds to participate in the study. Ethical clearance was obtained for this study from the Faculty of Veterinary Science, University of Pretoria (V043/14) to use dairy cows from five important dairy regions in South Africa.

### 2.2 Animals and sampling

#### 2.2.1 Animals

All animals in this study were kept under normal farm management conditions. There were no special treatments given to the animals.

#### 2.2.2 Sample size justification

Sensitivity was assumed to be 95% ( $S_N$ ) and it was desired to estimate this proportion with a precision of 5% and at a 95% level of confidence. The required sample size was calculated as 72 based on the following formula:

$$\text{Required sample size } (n) = Z^2_{1-\alpha/2} \times S_N \times (1 - S_N) / L^2 \quad (1)$$

Similar assumptions were made concerning the estimation of specificity resulting in the same sample size estimation (72). Since the pregnancy status of sampled cows was unknown until after sampling, it was also necessary to make an assumption concerning the expected pregnancy proportion in the average dairy cow being presented for pregnancy diagnosis. If 40% of the 72 cows are expected to be non-pregnant (open), then the minimum sample size of cows to sample is calculated as follows:

$$72 = n \times x, \text{ n is unknown and x is 0.4}$$

$$n = 72 / 0.4$$

$$= 180$$

Adding the potential losses (10 %) due to expected lost samples and labelling errors, the sample size for each herd was calculated as follows:

$$n = 180 + 10\%$$

$$= 198 \text{ each herd}$$

There were five veterinary practitioners, therefore the total sample size was estimated as 990 (198\*5) cows.

### 2.2.3 Sampling

The veterinary practitioners have done on-farm sampling and pregnancy examinations from July to September 2014. The cows were examined at 28-35 days after AI and followed up 14 days later (42-49 days after AI) if there were no subsequent signs of oestrus (Fig 1.1). These were designated the 'A' and 'B' samples, respectively. At each examination, cows were examined for pregnancy status and blood and milk samples were collected. Due to the limitations of accuracy of pregnancy diagnosis before day 35 and the fact that some practitioners were not used to examining animals this early, pregnancy results based on transrectal ultrasonography at the 'B' sampling ('TRUS B') were considered the reference test ('gold standard'). Sensitivity and specificity of the laboratory tests were estimated relative to these results.

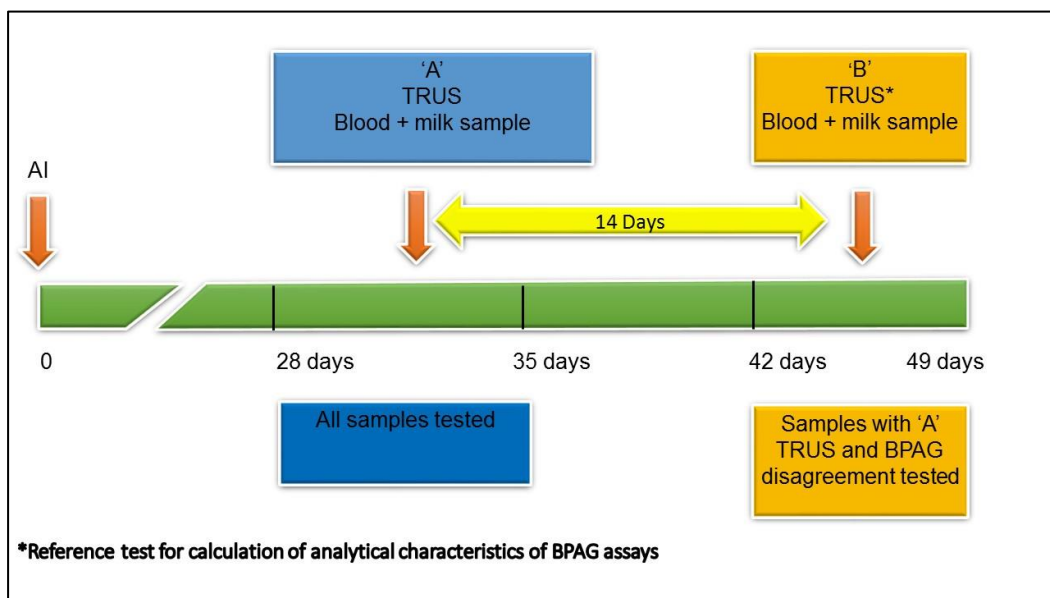


Figure 1.1: Graphical presentation of the study design.

The blood samples were collected from the coccygeal vein into plain evacuated 10 ml vacutainer tubes (BD Vacutainer®). Milk samples were collected from two teats and transferred into 11 ml tubes (brown-top tubes) which contained a commercial milk preservative (Broad Spectrum Microtabs II, Advanced Instruments, INC.). After collection, the samples were held at ambient temperature and shipped by overnight courier to the University of Pretoria, Onderstepoort Campus. The samples were processed at the Endocrine Research Laboratory within the Department of Anatomy and Physiology.

### 2.2.4 Pregnancy examination by transrectal ultrasonography

Equipment, methodology, and other factors regarding how the practitioners conducted examinations were as follows:

Pregnancies were diagnosed using two types ultrasound machines, which were equipped with 5.0 MHz or 7.5 MHz linear transducer arrays. Two practitioners used 5.0 MHz scanners and the other three used 7.5 MHz linear transducer arrays. Pregnancies were diagnosed by

visualization of membranes, embryo, foetus or an apparent heartbeat. During examination, cows were restrained using head gates, palpation rails, a crush area or at the milking parlour. The normal routines for examining pregnancy per practitioner ranges from 28 to 50 days after breeding. The experience level of the participating practitioners for diagnosing pregnancy using the ultrasound scanner ranges from less than a year to 8 years. The data collected was captured on either hand-written list or computer-generated lists.



Figure 1.2: Photograph of one of the five veterinary practitioners with a transrectal ultrasound used for pregnancy diagnosis.



Figure 1.3: Photograph of dairy cows lined up for pregnancy examination and sample collection.

#### 2.2.5 Sample processing and storage

The blood was centrifuged at  $1,821 \times g$  for 15 min and the sera were harvested into polystyrene tubes and aliquots were stored at  $-20^{\circ}\text{C}$  until analysis. Milk samples were stored at  $4^{\circ}\text{C}$  until analysis. The samples were only exposed to a single freeze-thaw cycle at room temperature



(18-25°C). The laboratory assays were performed using the commercially-available kits per the manufacturer's instructions. The samples were labelled with unique labels and laboratory technicians performing the ELISA tests were blinded to the pregnancy status of the cows.

## 2.2.6 Laboratory assays

### 2.2.6.1 IDEXX Bovine Pregnancy Kit©

The IDEXX Bovine Pregnancy Kit© (Bovine Pregnancy Test Kit, IDEXX Laboratories, Inc., Westbrook, Maine, USA) comes with the following reagents and items inside the box: Two 96-well plates, wash concentrate, sample diluent, positive and negative controls, detector solution, conjugate solution, substrate solution and stop solution. Before running the assay, all reagents were brought to room temperature (25°C). The reagents were mixed by gently swirling before using them. The wash concentrate was diluted 10-fold (1/10) with distilled water before use. The sample positions were recorded on an ELISA plate template sheet (Figure 1.4). The sample diluent of 25 µL was added into each well. One hundred µL of the positive control was added into each well and the same was done for the negative control. In each well, 100 µL of serum samples was added. The plate was covered and incubated for 60 minutes ( $\pm$  5 minutes) at 37 °C with platform rotating 450 rpm. The liquid contents were discarded into a waste reservoir after washing. The wells were filled with approximately 300 µL of 1X wash solution and washed 3 times with an automated washer (ThermoScientific, Model: Wellwash AC). The liquid contents were discarded into waste reservoir after washing. Immediately after washing, 100 µL of detector solution was added into each well.

The plate was covered and incubated for 30 minutes at room temperature (25°C). After incubation, the plate was washed 3 times. One hundred µL of horseradish peroxidase conjugate (HRPO) solution was added into each well. The plate was covered and incubated for 30 minutes at room temperature. After incubation, the plate was washed a single time followed by the addition of 100 µL of 3, 3', 5, 5' tetramethylbenzidine substrate (TMB). Colour development was allowed to occur for 15 minutes with incubation done at room temperature. After incubation, 100 µL of stop solution was added into each well to stop the reaction. The samples and controls were measured at A (450 nm) – A (REF). The reference wavelength (REF) was measured at 620 nm. Therefore, the test samples were calculated as sample minus negative control mean (S-N) with the following equation:

$$S-N = \text{Sample A (450 nm - REF)} - NC\bar{x}$$

Where Sample A (450 nm REF) is the sample result and  $NC\bar{x}$  is the corrected negative control mean.

Pregnancy results were determined based on the cut-off values determined by the BPAG A ELISA manufacturer. For the serum BPAG A ELISA, if the S-N value was  $<0.300$ , the animal was classified as negative (not pregnant) and if the S-N value was  $\geq 1.00$ , the animal was classified as positive (pregnant). If the S-N value was between  $>0.3$  and  $<1$ , the animal is

classified as a recheck. The results were read with plate reader (BioTek, Model EL800) linked to computer that is equipped with a software (xCheckPlus® Software).

#### 2.2.6.2 IDEXX Milk Pregnancy Kit©

The IDEXX Milk Pregnancy Kit© (Milk Pregnancy Test Kit, IDEXX Laboratories, Inc., Westbrook, Maine, USA) comes with the following reagents and items inside the box: Two 96-well plates, wash concentrate, sample diluent, positive and negative controls, detector solution, conjugate solution, substrate solution and stop solution. Before running the assay, all reagents were brought to room temperature (25°C). The reagents were mixed by gently swirling before using them. The wash concentrate was diluted 10-fold (1/10) with distilled water before use. The sample positions were recorded on an ELISA plate template sheet (Figure 1.4). The positive and negative controls were also added to each test plate. In appropriate wells, 150 µL of milk sample was added into each well. The plate was covered and incubated for 120 minutes at 37°C with the platform rotating 450 rpm. The plate was covered tightly sealed with adhesive cover to avoid evaporation. The liquid contents were discarded into a waste reservoir. The plate was washed 4 times with an automated washer (ThermoScientific, Model: Wellwash AC) with approximately 300 µL wash solution. After washing, 100 µL of detector solution was added into each well.

The plate was covered with adhesive covers and incubated for 30 minutes at room temperature (25°C). After incubation, the liquid contents were discarded and washed 4 times. After washing, 100 µL of horseradish peroxidase conjugate (HRPO) was added into each well. The plate was covered and incubated for 30 minutes at room temperature. The liquid contents were discarded and washed 4 times. After washing, 100 µL of 3, 3', 5, 5'-tetramethylbenzidine substrate (TMB) were added into each well for colour development. The plate was incubated for 20 minutes at room temperature. After incubation, 100 µL of stop solution was added into each well to stop the reaction. The samples and controls were measured as A (450 nm) – A (REF). The reference wavelength (REF) was measured at 620 nm. Therefore, the test samples were calculated as sample minus negative control mean (S-N) with the following equation:

$$S-N = \text{Sample A (450 nm - REF) - NCx}$$

Pregnancy results were determined based on the cut-off values determined by the BPAG A ELISA manufacturer. For the milk BPAG A ELISA, if the S-N value was <0.100 the animal was classified as negative. If the S-N value was  $\geq 0.250$ , the animal was classified positive. If the S-N value was between >0.1 and <0.25, the animal was classified as a recheck. The results were read with plate reader (BioTek, Model EL800) linked to computer that is equipped with a software (xCheckPlus® Software).

#### 2.2.6.3 IDEXX Visual Pregnancy Kit©

The IDEXX Visual Pregnancy Kit© (Visual Pregnancy Test Kit, IDEXX Laboratories, Inc., Westbrook, Maine, USA) comes with the following reagents and items inside the box: Two 96-

well plates, precision pipettes and tips, wash concentrate, sample diluent, positive and negative controls, detector solution, conjugate solution, substrate solution and stop solution. The items that were not included in the kit were the cover sheets and the wash bottle. Before doing the assay, all the reagents were brought to room temperature (25°C). The reagents were mixed gently by swirling before using them. The wash concentration was diluted 10-fold (1/10) with distilled water before use. To start doing the assay, a drop of sample diluent was added in all the wells. Then an undiluted positive and negative controls were added into wells. Using a precision pipette, sera were added to the remaining wells (Fig 1.4). The 96-well plate was mixed by gently tapping it, covered and incubated for 30 minutes at room temperature. After incubation, the plate was inverted over a waste reservoir to discard the liquid contents. The plate was filled with a wash solution and washed 3 times with a wash bottle. After washing, two drops detector solution was added in all the wells.

The plate was covered and incubated for 30 minutes at room temperature. After incubation, the plate was washed 3 times. Two drops of horseradish peroxidase conjugate (HRPO) solution were added into each well after drying. The plate was then covered and incubated for 30 minutes at room temperature. After incubation, the plate was washed 3 times. Immediately after washing, two drops of 3, 3', 5, 5' tetramethylbenzidine substrate (TMB) solution was added into each well. The plate was covered and incubated for 15 minutes at room temperature to allow colour development. After incubation, two drops of stop solution were added into each well and the colour development determined visually.

|   | 1  | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|---|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | +  | A5  | A13 | A21 | A29 | A37 | A45 | A53 | A61 | A69 | A77 | A85 |
| B | +  | A6  | A14 | A22 | A30 | A38 | A46 | A54 | A62 | A70 | A78 | A86 |
| C | -  | A7  | A15 | A23 | A31 | A39 | A47 | A55 | A63 | A71 | A79 | A87 |
| D | -  | A8  | A16 | A24 | A32 | A40 | A48 | A56 | A64 | A72 | A80 | A88 |
| E | A1 | A9  | A17 | A25 | A33 | A41 | A49 | A57 | A65 | A73 | A81 | A89 |
| F | A2 | A10 | A18 | A26 | A34 | A42 | A50 | A58 | A66 | A74 | A82 | A90 |
| G | A3 | A11 | A19 | A27 | A35 | A43 | A51 | A59 | A67 | A75 | A83 | A91 |
| H | A4 | A12 | A20 | A28 | A36 | A44 | A52 | A60 | A68 | A76 | A84 | A92 |

Figure 1.4: Representation of positive (blue) and negative control (clear), serum or milk (yellow) sample positions in a 96-well plate.

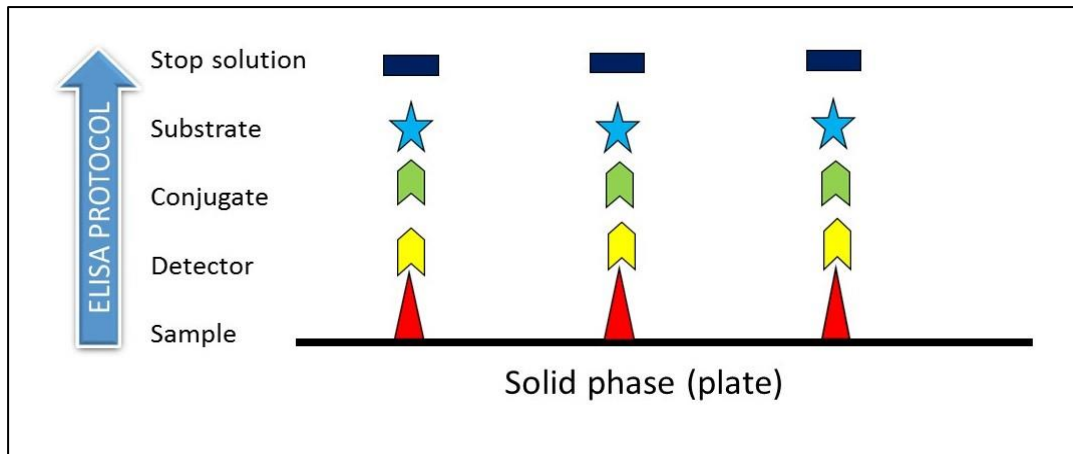


Figure 1.5: Graphical representation of Indirect ELISA protocol.

### 2.3 Statistical analysis

To evaluate pregnancy results from the serum, milk and serum visual BPAG A ELISA tests in cows of unknown pregnancy status, 2 x 2 contingency tables were constructed to calculate sensitivity and specificity for the serum, milk and serum visual BPAG A ELISA tests 28-35 days after AI. The reference standard for estimating sensitivity and specificity was transrectal ultrasonography at 42-49 days after breeding (TRUS B).

Sensitivity (Se) was estimated as the proportion of truly pregnant cows divided by a total of truly pregnant and false-negative cows multiply by 100%. The specificity (Sp) was estimated as the proportion of truly non-pregnant cows divided by a total of truly non-pregnant and false-positive cows multiply by 100%.

Binary logistic regression was used to determine the effects of breed (Holstein, Jersey and Crossbred), lactation number (1-9), days after AI (28-35), days in milk (DIM) on sensitivity and specificity for the three BPAG A tests. All the co-variates were considered as categories. Within SPSS Statistics, click variable view, days after AI were categorised with values and labels as follows: (1) 28, (2) 29, (3) 30, (4) 31, (5) 32, (6) 33, (7) 34 and (8) 35 days after AI. The measure of the data was considered as nominal. The breed types were categorised as follows: Breed (1) = Holstein, Breed (2) = Jersey and Breed (3) = Crossbred. The measure of the breed data was considered as ordinal. The lactation stages were binned (visual binning) into four categories: Lactation 1, 2, 3 and 4+. The data for days in milk was categorised into four categories by visual binning within SPSS Statistic as follows: DIM (1) =  $\leq 109$ , DIM (2) = 110-145, DIM (3) = 146-216 and DIM (4) = 217+.

The serum, milk and serum visual BPAG A ELISA test result for truly pregnant cows was the dependent variable for the evaluation of effects on sensitivity. Breeds, lactation number, days after AI and DIM were included as co-variates in each model.

The serum, milk and serum visual BPAG A ELISA test result for truly non-pregnant cows was the dependent variable for evaluating the effects on specificity estimates. Breeds, lactation number, days after AI and DIM were included as co-variates in each model.

The effect of breed, lactation number, days after AI and DIM on serum and milk BPAG A optical density (OD) values were determined independently using general linear model (GLM) approach. Pregnancy-associated glycoprotein OD S-N values for serum and milk were the dependent variables and breed, lactation number, days after AI and DIM were included as co-variates.

Before statistical analysis for serum and milk BPAG A levels, normality of the data set was checked with descriptive statistics and a histogram obtained within SPSS® Statistics. Within the SPSS Statistics, click 'analyse', click 'descriptive statistics', click 'explore'. The serum and milk BPAG A levels were considered as dependent variables. Click 'plots' and a new window pops out, uncheck everything for descriptive and check 'normality plots with tests'. The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to check for normality considering the p-value. If  $p < 0.005$ , then accept the null hypothesis and conclude that the data comes from non-normal distribution. If  $p > 0.005$ , then reject the alternative hypothesis and conclude that the data comes from normal distribution. If the data was not coming from a normal distribution, data was transformed. Within the SPSS Statistics, click 'transform', click 'rank cases', the serum or milk A BPAG levels were selected as variables. Click 'rank types' and check 'rank' and click 'continue'. The ranks for serum and milk A BPAG levels were ranked from smallest to largest values.

Pregnancy loss was defined as cows with pregnant results at all the BPAG A ELISA A and TRUS A results and non-pregnant results at TRUS B examination. Binary logistic regression model procedure was used to determine the effect of breed, lactation number, days after AI and DIM on the incidence of pregnancy loss. Pregnancy loss was the dependent variable and breeds, lactation number, days after AI and DIM were included as co-variates. Statistical analysis was performed using commercial software (IBM® SPSS Statistic, version 23) and statistical significance was set  $P < 0.05$ .

## CHAPTER THREE: RESULTS

### 3.1 Test performance of the serum, milk, and serum visual BPAG A ELISA relative to TRUS

The results for number of blood and milk samples collected and those eligible for analysis with complete data per practitioner is presented in Table 3.1. The total number of samples collected in this study were 1197 and samples eligible for analysis were 1031. A total of 166 samples were received after 25 days due to being delivered at the wrong address. These samples were discarded due to longer storage in an unsuitable temperature to process and analyse for the assays.

Table 3.1: Cattle sampled per veterinary practitioner

| Practice | No. of animals sampled | Eligible animals with complete data |
|----------|------------------------|-------------------------------------|
| A        | 257                    | 223                                 |
| B        | 226                    | 166                                 |
| C        | 241                    | 240                                 |
| D        | 248                    | 221                                 |
| E        | 225                    | 181                                 |
| Total    | 1197                   | 1031                                |

The descriptive results of the breed type (Holstein, Jersey and cross), days in milk (DIM), lactation number and days after AI per practitioner are presented in Table 3.2. Days in milk was categorised into four categories: (1)  $\leq 109$ , (2) 110-145, (3) 146-216 and (4) 217+ for analysis (not shown in Table). The lactation number was categorised into four categories: (1) 1<sup>st</sup> lactation, (2) 2<sup>nd</sup> lactation, (3) 3<sup>rd</sup> lactation and (4)  $\geq 4^{\text{th}}$  lactation (not shown in Table). Days after AI after AI was from 28 to 35 days of gestation.

Table 3.2: Descriptive statistics for breed, days in milk, lactation number and days after AI per veterinary practice

| Practice | Variable         | n   | Proportion (95% CI) ** | Mean $\pm$ SD*     |
|----------|------------------|-----|------------------------|--------------------|
| A        | Breed            |     |                        |                    |
|          | Holstein         | 108 | 52.9 (46.07-59.75)     | -                  |
|          | Jersey           | 43  | 21.1 (15.89-27.08)     | -                  |
|          | Crossbred        | 53  | 26.0 (20.31-32.33)     | -                  |
|          | DIM#             | 32  | -                      | 170.53 $\pm$ 81.85 |
|          | Lactation number | 204 | -                      | 2.4 $\pm$ 1.38     |
|          | Days after AI    | 204 | -                      | 31.53 $\pm$ 2.00   |
| B        | Breed            |     |                        |                    |
|          | Holstein         | 222 | 100.0 (98.66-100.0)    | -                  |
|          | Jersey           | 0   | 0                      | -                  |
|          | Crossbred        | 0   | 0                      | -                  |
|          | DIM              | 165 | -                      | 168.43 $\pm$ 83.15 |
|          | Lactation number | 166 | -                      | 2.66 $\pm$ 1.36    |
|          | Days after AI    | 166 | -                      | 31.63 $\pm$ 1.90   |
| C        | Breed            |     |                        |                    |
|          | Holstein         | 240 | 100.0 (98.76-100.0)    | -                  |
|          | Jersey           | 0   | 0                      | -                  |
|          | Crossbred        | 0   | 0                      | -                  |
|          | DIM              | 240 | -                      | 167.23 $\pm$ 80.34 |
|          | Lactation number | 240 | -                      | 2.51 $\pm$ 1.40    |
|          | Days after AI    | 240 | -                      | 31.66 $\pm$ 1.98   |
| D        | Breed            |     |                        |                    |
|          | Holstein         | 72  | 72.0 (71.99-82.94)     | -                  |
|          | Jersey           | 65  | 37.0 (23.69-35.67)     | -                  |
|          | Crossbred        | 0   | 0                      | -                  |
|          | DIM              | 221 | -                      | 166.04 $\pm$ 84.23 |
|          | Lactation number | 33  | -                      | 2.63 $\pm$ 1.26    |
|          | Days after AI    | 221 | -                      | 31.69 $\pm$ 1.82   |
| E        | Breed            |     |                        |                    |
|          | Holstein         | 173 | 100.0 (98.28-100.0)    | -                  |
|          | Jersey           | 0   | 0                      | -                  |
|          | Crossbred        | 0   | 0                      | -                  |
|          | DIM              | 173 | -                      | 170.20 $\pm$ 84.28 |
|          | Lactation number | 173 | -                      | 2.30 $\pm$ 1.35    |
|          | Days after AI    | 173 | -                      | 31.53 $\pm$ 2.01   |

n: number or proportion of cows

\*SD: Standard deviation

\*\*95% CI: 95% Confidence Intervals

#: Days in milk

The total number of pregnant and non-pregnant cows based on transrectal ultrasonography was 534 and 400, respectively. The pregnancy rate was 57, 2% (534/934) based on transrectal ultrasonography at 42 to 49 days after AI.

The test performance of the serum BPAG A ELISA was evaluated on a total of 928 samples, these being for which a complete data set was available. The results are presented in Table 3.3. The serum BPAG A ELISA tested 527 animals as positive and 229 as negative. The serum BPAG A ELISA estimated a sensitivity of 99.8% and specificity of 57.3%. The total numbers of false positive and negative results were 171 and 1, respectively.

Table 3.3: Summary of contingency data for evaluation of <sup>1</sup>sensitivity and <sup>2</sup>specificity of the serum BPAG A ELISA for determining pregnancy status at 28-35 days compared to TRUS B

| BPAG A ELISA | TRUS B   |              |             |
|--------------|----------|--------------|-------------|
|              | Pregnant | Not pregnant | Grand total |
| Positive     | 527 (a)  | 171 (b)      | 698         |
| Negative     | 1 (c)    | 229 (d)      | 230         |
| Grand total  | 528      | 400          | 928 (n)     |

<sup>1</sup>Proportion of samples from pregnant cows with a positive BPAG A ELISA,  $[a / (a+c)] \times 100$ .

<sup>2</sup>Proportion of samples from non-pregnant cows with a negative BPAG A ELISA,  $[d / (b+d)] \times 100$ .

The test performance of the milk BPAG A ELISA was evaluated on a total of 927 samples and is presented in Table 3.4. The milk BPAG A ELISA tested 526 animals as positive and 220 as negative. The milk BPAG A ELISA estimated a sensitivity of 99.4% and specificity of 55.3%. The total number of false positives and negatives were 178 and 3, respectively.



Table 3.4: Summary of contingency data for evaluation <sup>1</sup>sensitivity and <sup>2</sup>specificity of the milk BPAG A ELISA test for determining pregnancy status at 28-35 days compared to TRUS B

| TRUS B       |          |              |             |
|--------------|----------|--------------|-------------|
|              | Pregnant | Not pregnant | Grand total |
| BPAG A ELISA |          |              |             |
| Positive     | 526 (a)  | 178 (b)      | 704         |
| Negative     | 3 (c)    | 220 (d)      | 223         |
| Grand total  | 529      | 398          | 927 (n)     |

<sup>1</sup>Proportion of samples from pregnant cows with a positive BPAG A ELISA,  $[a / (a+c)] \times 100$ .

<sup>2</sup>Proportion of samples from non-pregnant cows with a negative BPAG A ELISA,  $[d / (b+d)] \times 100$ .

The test performance of the serum visual BPAG A ELISA was evaluated on a total of 925 samples and is presented in Table 3.5. The visual BPAG A ELISA tested 525 animals as positive and 219 as negative. The total number of false positives and negatives were 179 and 2, respectively. The visual BPAG A ELISA has sensitivity of 99.6% and specificity of 55.0%.

Table 3.5: Summary of contingency data for evaluation of <sup>1</sup>sensitivity and <sup>2</sup>specificity of serum visual BPAG A ELISA test for determining pregnancy status at 28-35 days compared to TRUS B

| TRUS B       |          |              |             |
|--------------|----------|--------------|-------------|
|              | Pregnant | Not pregnant | Grand total |
| BPAG A ELISA |          |              |             |
| Positive     | 525 (a)  | 179 (b)      | 704         |
| Negative     | 2 (c)    | 219 (d)      | 221         |
| Grand total  | 527      | 398          | 925 (n)     |

<sup>1</sup>Proportion of samples from pregnant cows with a positive BPAG A ELISA,  $[a / (a+c)] \times 100$ .

<sup>2</sup>Proportion of samples from non-pregnant cows with a negative BPAG A ELISA,  $[d / (b+d)] \times 100$ .

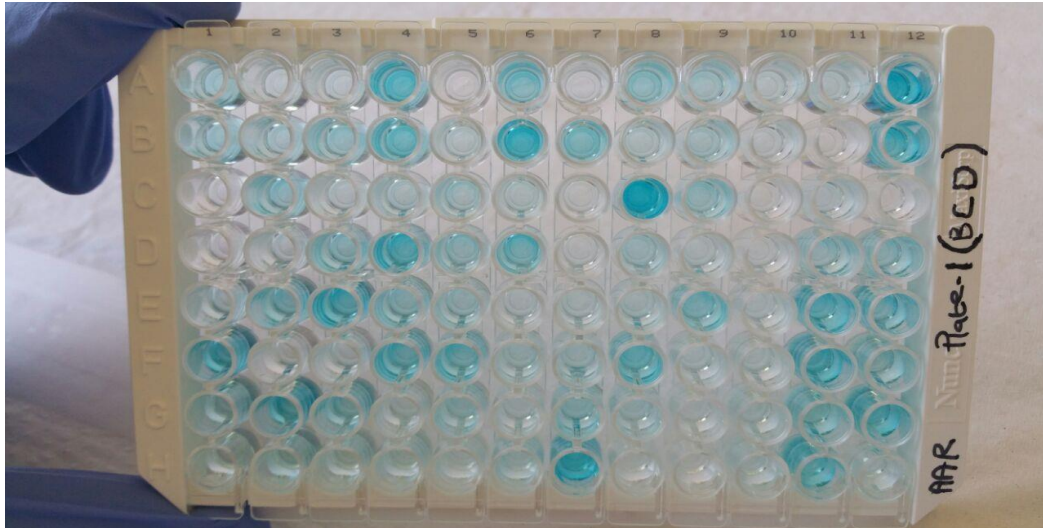


Figure 3.1: A photograph of the serum visual BPAG A ELISA showing positive (blue) and negative (clear).

The estimates for sensitivity and specificity, proportion of false positive and -negatives of serum, milk and serum visual BPAG A ELISA assays are summarized in Table 3.6. In this study, sensitivity ranged from 99.4 to 99.8% and specificity ranged from 55.0 to 57.3% for the serum, milk and serum visual BPAG A ELISA tests.

Table 3.6: Sensitivity, specificity, proportion of false positive and –negative of serum, milk, and serum visual BPAG A ELISA tests for determination of pregnancy status 28-35 d

|                     | Sensitivity<br>[% (95% CI <sup>1</sup> )] | Specificity<br>[% (95% CI)] |
|---------------------|---|-----------------------------|
| <b>BPAG A ELISA</b> |   |                             |
| Serum               | 99.8 [99.07-99.99]                        | 57.3 [52.36-62.04]          |
| Milk                | 99.4 [98.46-99.86]                        | 55.3 [50.36-60.11]          |
| Visual              | 99.6 [98.75-99.94]                        | 55.0 [50.11-59.87]          |

<sup>1</sup>CI= Confidence interval

### 3.2 Factors influencing sensitivity and specificity of serum, milk, and serum visual BPAG ELISAS

Logistic regression indicated a significant influence of days after AI and breed on the specificity of the serum, milk, and serum visual BPAG A ELISA. However, there was no significant influence of days in milk, lactation number, days after AI and breed on the sensitivity of the serum, milk and serum visual BPAG A ELISA (Appendix).

The analysis of false-positives for the serum BPAG A ELISA is presented in Table 3.7. The results showed that days after AI had an influence ( $p < 0.05$ ) at 28 and 29 days after AI on the false-positives of the serum BPAG A ELISA. Cows that were examined at 28 days after AI were four times less likely to have a false-positive reaction in the serum ELISA test compared to

cows examined at 35 days after AI (OR = 0.250; P = 0.013). Cows that were examined at 29 days after AI were 2.9 times less likely to have a false-positive reaction in the serum BPAG A ELISA compared to cows examined at 35 days after AI (OR = 0.345; P = 0.027). Holstein cows were 16.9 times less likely to have a false-positive reaction in the serum BPAG A ELISA compared to Jersey cows (OR = 0.059; P = 0.0001).

Table 3.7: Effect of days after AI and breed on false-positives serum BPAG A ELISA

| Variable              | Parameter estimate (B) | P-value (Wald) | Odds ratio (95% CI) |
|-----------------------|------------------------|----------------|---------------------|
| <b>Days after AI</b>  |                        |                |                     |
| 28                    | -1.386                 | 0.013          | 0.250 (0.084-0.748) |
| 29                    | -1.065                 | 0.027          | 0.345 (0.134-0.885) |
| 30                    | -0.649                 | 0.166          | 0.523 (0.209-1.310) |
| 31                    | -0.288                 | 0.516          | 0.750 (0.135-1.785) |
| 32                    | -0.609                 | 0.179          | 0.544 (0.224-1.322) |
| 33                    | -0.470                 | 0.335          | 0.625 (0.240-1.624) |
| 34                    | -0.342                 | 0.498          | 0.711 (0.265-1.908) |
| 35                    | Reference              | -              | -                   |
| <b>Breed category</b> |                        |                |                     |
| Cross                 | -0.323                 | 0.739          | 0.724 (0.108-4.839) |
| Holstein              | -2.828                 | 0.0001         | 0.059 (0.018-0.198) |
| Jersey                | Reference              | -              | -                   |

The analysis of false-positives for the milk BPAG A ELISA is presented in Table 3.8. The results showed that days after AI had an influence ( $p < 0.05$ ) at 28 and 29 days after AI on the false-positives of the milk BPAG A ELISA. Cows that were examined at 28 days after AI were 3.4 times less likely to have a false-positive reaction in the milk ELISA test compared to cows examined at 35 days after AI (OR = 0.294; P = 0.024). Cows that were examined at 29 days after AI were 3.9 times less likely to have a false-positive reaction in the milk BPAG A ELISA compared to cows examined at 35 days after AI (OR = 0.255; P = 0.006). Holstein cows were 16.9 times less likely to have a false-positive reaction in the milk BPAG A ELISA compared to Jersey cows (OR = 0.063; P = 0.0001).

Table 3.8: Effect of days after AI and breed on false-positives on milk BPAG A ELISA

| Variable              | Parameter estimate (B) | P-value (Wald) | Odds ratio (95% CI)   |
|-----------------------|------------------------|----------------|-----------------------|
| <b>Days after AI</b>  |                        |                |                       |
| 28                    | -1.224                 | 0.024          | 0.294 (0.101-0.853)   |
| 29                    | -1.365                 | 0.006          | 0.255 (0.097-0.671)   |
| 30                    | -0.507                 | 0.281          | 0.602 (0.240-1.515)   |
| 31                    | -0.385                 | 0.389          | 0.680 (0.283-1.633)   |
| 32                    | -0.639                 | 0.162          | 0.528 (0.216-1.291)   |
| 33                    | -0.618                 | 0.209          | 0.539 (0.206-1.412)   |
| 34                    | -0.547                 | 0.285          | 0.579 (0.213-1.576)   |
| 35                    | Reference              | -              | -                     |
| <b>Breed category</b> |                        |                |                       |
| Cross                 | 18.934                 | 0.998          | 167118087.191 (0.000) |
| Holstein              | -2.758                 | 0.0001         | 0.063 (0.0190-0.212)  |
| Jersey                | Reference              | -              | -                     |

The analysis of false-positives for the serum visual BPAG A ELISA is presented in Table 3.9. The results showed that days after AI had an influence ( $p < 0.05$ ) at 28 and 29 days after AI on the false-positives of the serum visual BPAG A ELISA. Cows that were examined at 28 days after AI were 3.4 times less likely to have a false-positive reaction in the serum visual ELISA test compared to cows examined at 35 days after AI (OR = 0.294;  $P = 0.024$ ). Cows that were examined at 29 days after AI were 2.6 times less likely to have a false-positive reaction in the serum visual BPAG A ELISA compared to cows examined at 35 days after AI (OR = 0.381;  $P = 0.044$ ). Holstein cows were 16.9 times less likely to have a false-positive reaction in the serum visual BPAG A ELISA compared to Jersey cows (OR = 0.067;  $P = 0.0001$ ).

Table 3.9: Effect days after AI and breed on false-positives serum visual BPAG A ELISA

| Variable              | Parameter estimate (B) | P-value (Wald) | Odds ratio (95% CI)   |
|-----------------------|------------------------|----------------|-----------------------|
| <b>Days after AI</b>  |                        |                |                       |
| 28                    | -1.224                 | 0.024          | 0.294 (0.101-0.853)   |
| 29                    | -0.966                 | 0.044          | 0.381 (0.149-0.973)   |
| 30                    | -0.796                 | 0.092          | 0.451 (0.179-1.139)   |
| 31                    | -0.410                 | 0.360          | 0.664 (0.276-1.596)   |
| 32                    | -0.698                 | 0.127          | 0.498 (0.203-1.219)   |
| 33                    | -0.575                 | 0.244          | 0.563 (0.214-1.479)   |
| 34                    | -0.381                 | 0.453          | 0.683 (0.252-1.848)   |
| 35                    | Reference              | -              | -                     |
| <b>Breed category</b> |                        |                |                       |
| Cross                 | 18.969                 | 0.998          | 173086590.305 (0.000) |
| Holstein              | -2.704                 | 0.0001         | 0.067 (0.020-0.225)   |
| Jersey                | Reference              | -              | -                     |

The additional analysis of the effect of days after AI on sensitivity and specificity of the serum, milk and serum visual BPAG A ELISA is presented in Table 3.10. Days after AI had an influence on the specificity of the serum, milk and serum visual BPAG A ELISA. However, days after AI did not have influence on the sensitivity of the serum, milk and serum visual BPAG A ELISA. The specificity of the serum, milk and serum visual BPAG A ELISA decreased from 29 days after AI (Fig. 3.4, 3.5 and 3.6). The specificity of the serum, milk and serum visual was higher in a Holstein cows than in Jersey and crossbred cows (Fig. 3.7).

Table 3.10: Effect of days after AI on sensitivity and specificity of the serum, milk, and serum visual BPAG A ELISA

| BPAG A ELISA  |        |        |        |        |              |       |
|---------------|--------|--------|--------|--------|--------------|-------|
| Days after AI | Serum  |        | Milk   |        | Serum visual |       |
|               | Se (%) | Sp (%) | Se (%) | Sp (%) | Se (%)       | Sp(%) |
| 28            | 100.0  | 75.0   | 100.0  | 69.0   | 97.0         | 69.0  |
| 29            | 100.0  | 69.0   | 98.4   | 72.0   | 100.0        | 69.2  |
| 30            | 100.0  | 59.0   | 100.0  | 52.0   | 100.0        | 59.0  |
| 31            | 100.0  | 50.0   | 100.0  | 49.0   | 100.0        | 49.3  |
| 32            | 100.0  | 58.0   | 100.0  | 55.0   | 100.0        | 57.0  |
| 33            | 100.0  | 55.0   | 100.0  | 55.0   | 100.0        | 53.4  |
| 34            | 98.0   | 51.3   | 98.0   | 53.0   | 98.0         | 49.0  |
| 35            | 100.0  | 43.0   | 98.3   | 39.2   | 100.0        | 39.2  |

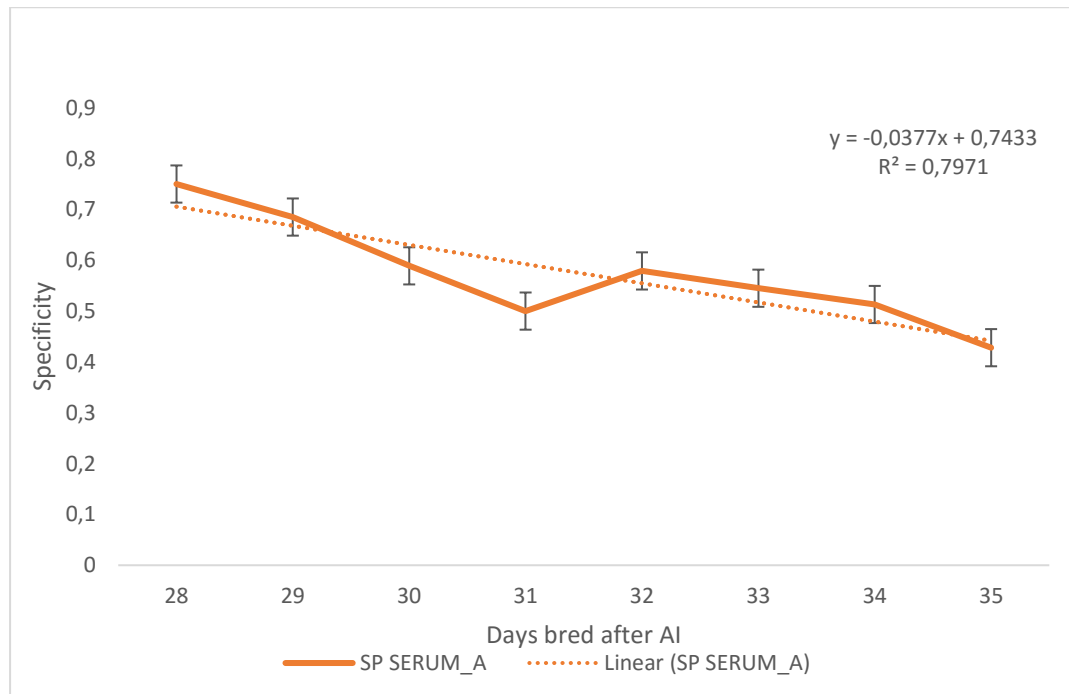


Figure 3.2: Effect of days after AI on specificity of the serum BPAG A ELISA.

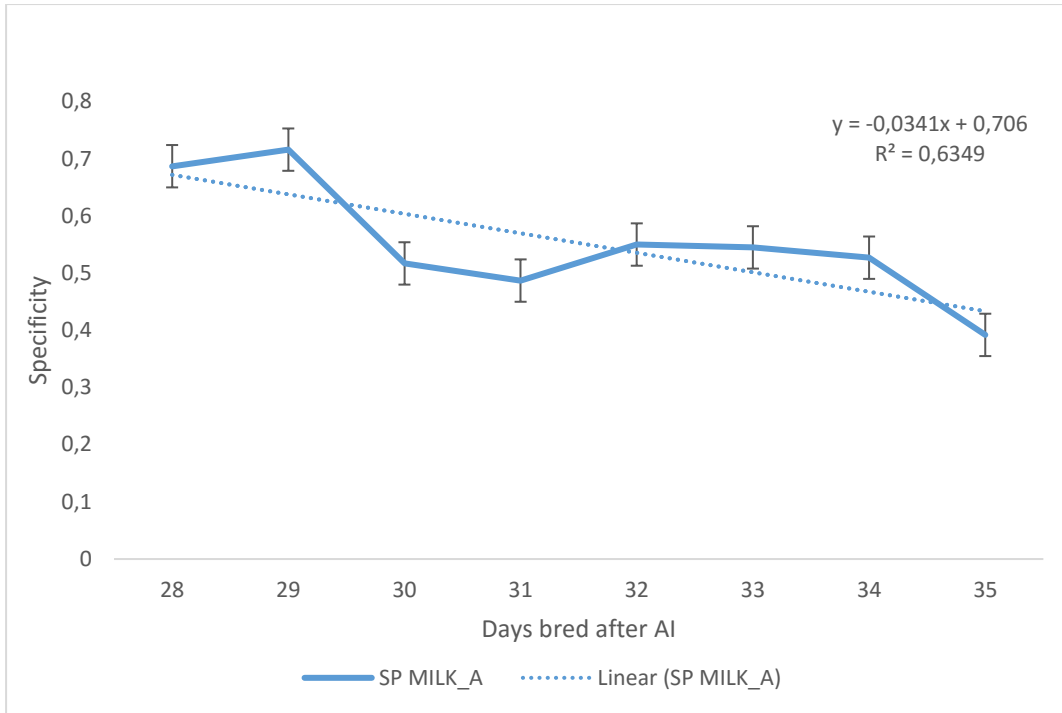


Figure 3.3: Effect of days after AI on specificity of the milk BPAG A ELISA.

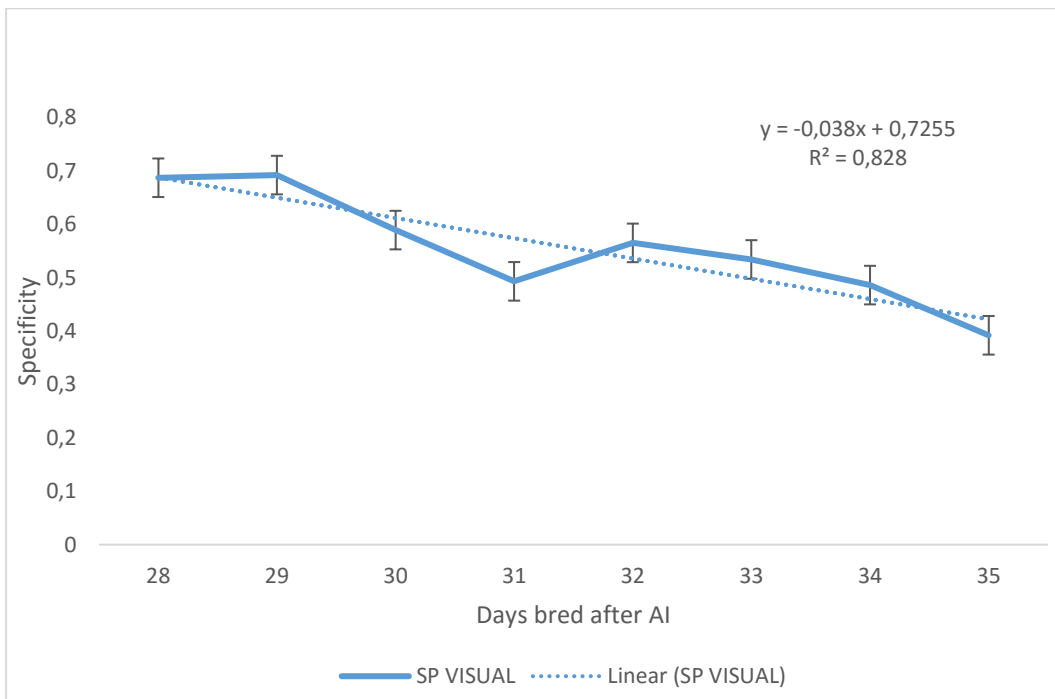


Figure 3.4: Effect of days after AI on specificity of serum visual BPAG A ELISA.

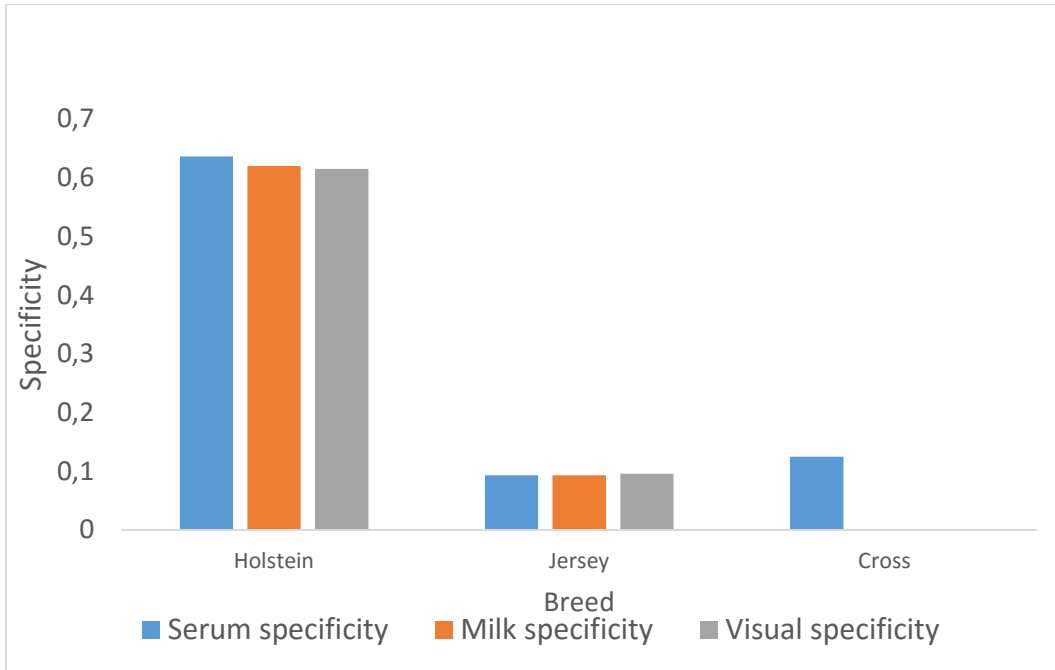


Figure 3.5: Effect of breed on specificity of serum, milk, and serum visual BPAG A ELISA.

### 3.3 Serum and milk BPAG A levels of pregnant cows based TRUS B

Table 3.11: Descriptive statistics for serum BPAG A ELISA levels before ranking

|          |        |
|----------|--------|
| Range    | 4.015  |
| Minimum  | -0.160 |
| Maximum  | 3.999  |
| Mean     | 1.159  |
| SD*      | 1.483  |
| Variance | 2.201  |
| Skewness | 0.724  |
| Kurtosis | -1.240 |

\*SD: Standard deviation



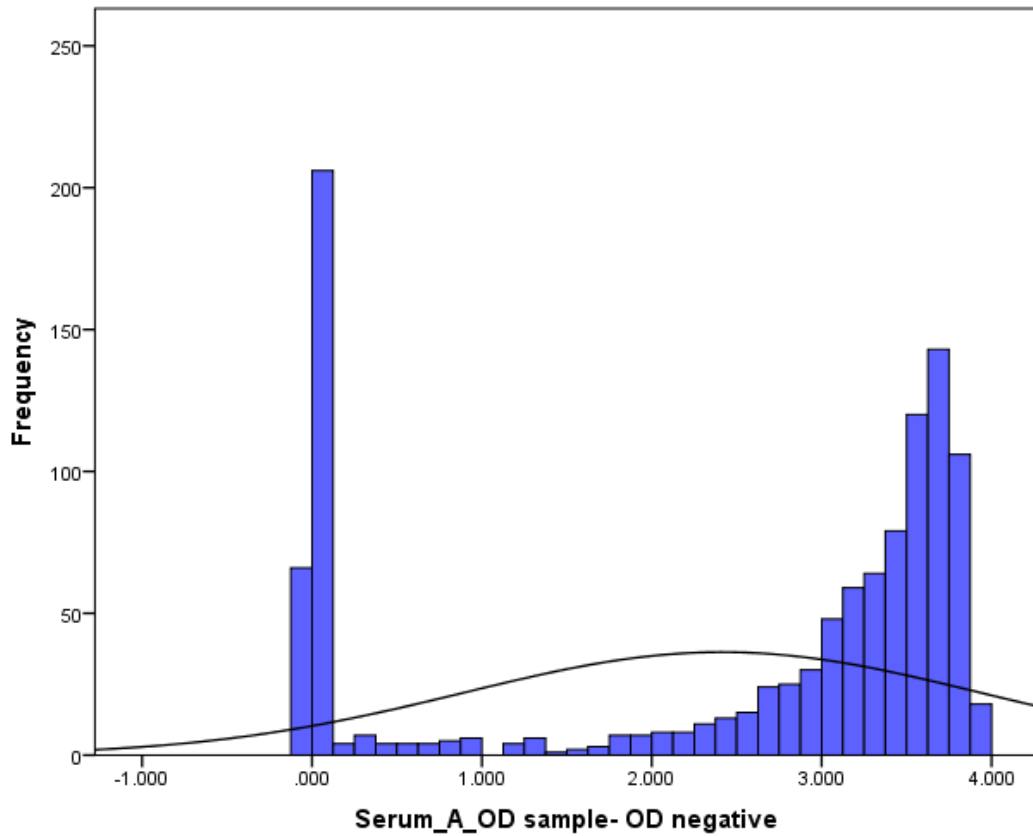


Figure 3.6: Histogram showing the distribution of serum BPAG A levels before ranking.

Table 3.12: Descriptive statistics for serum BPAG A ELISA levels before ranking

|          |        |
|----------|--------|
| Range    | 4.010  |
| Minimum  | -0.040 |
| Maximum  | 3.970  |
| Mean     | 0.877  |
| SD*      | 0.714  |
| Variance | 0.510  |
| Skewness | 0.641  |
| Kurtosis | 0.280  |

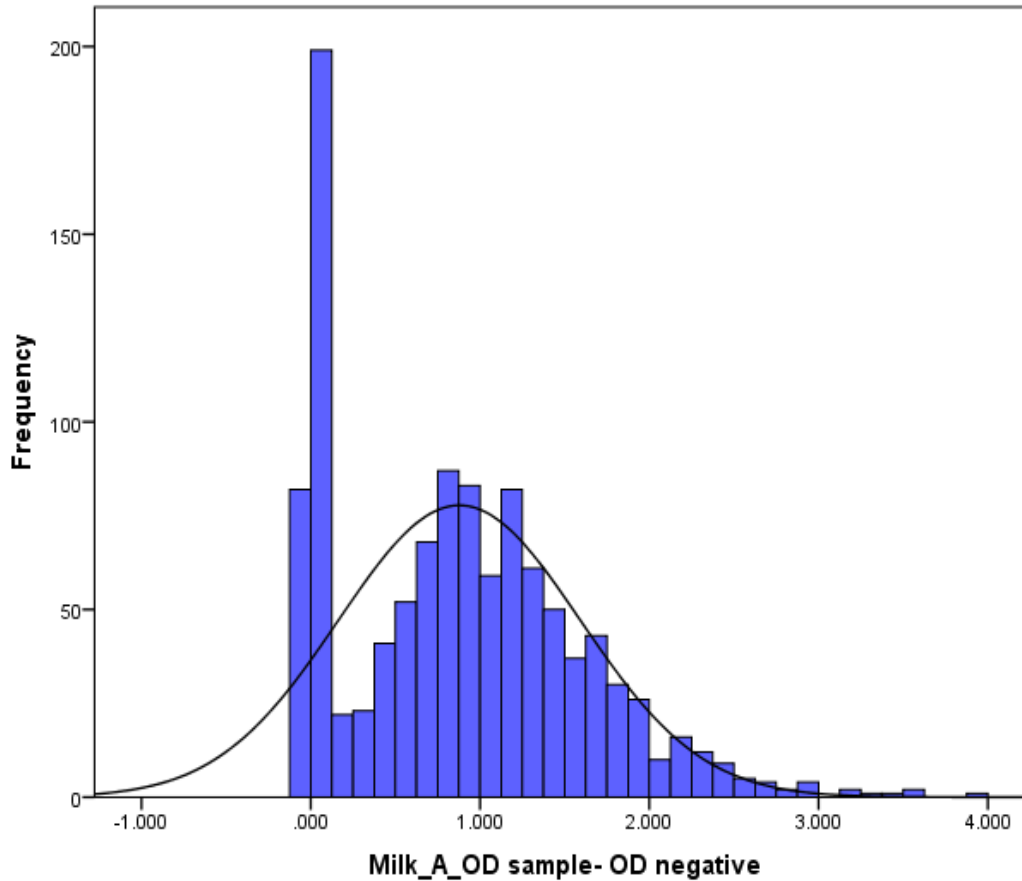


Figure 3.7: Histogram showing distribution of milk BPAG A levels before ranking.

An additional correlation analysis was conducted to compare OD sample-OD negative values from serum and milk BPAG A ELISA tests of the same cows (n=928) (Fig. 3.8). Overall, OD sample-OD sample negative values between the serum and milk BPAG A ELISA were highly correlated ( $R^2 = 0.64$ ) and the slope of the regression line reflects the greater relative serum BPAG A levels compared to milk BPAG A levels.

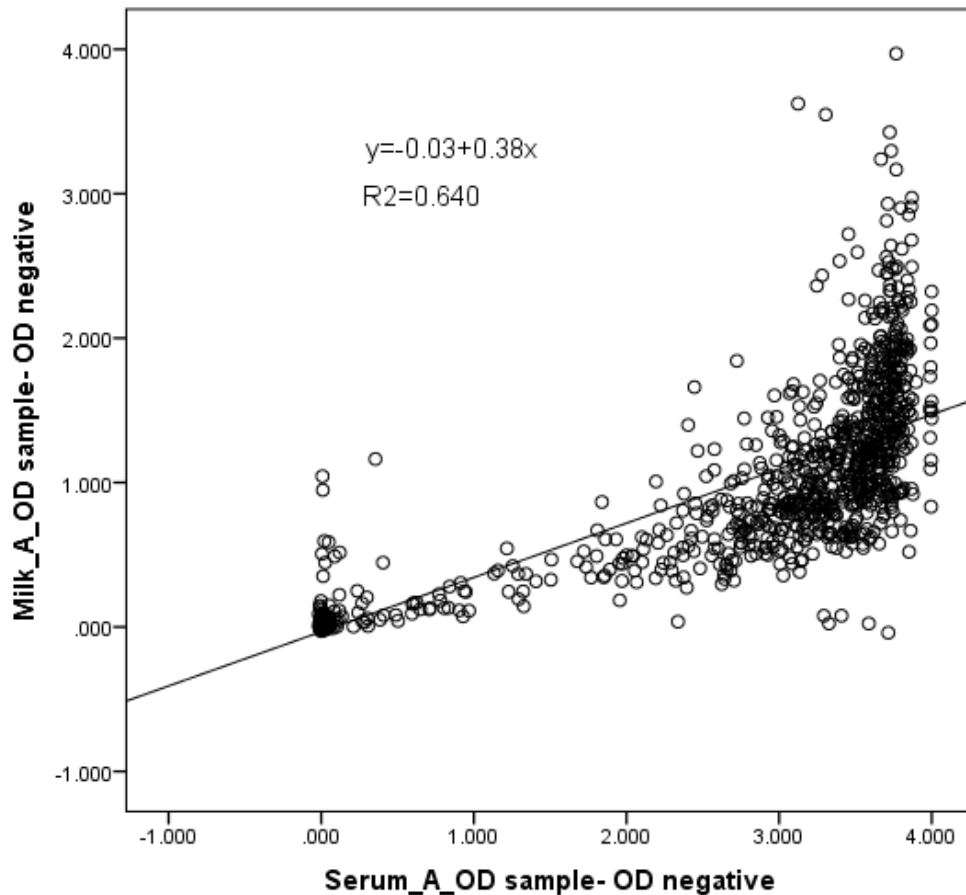


Figure 3.8: Relationship between relative levels of pregnancy-associated glycoproteins (BPAG) in serum and milk of cows from 28 to 35 days after AI.

### 3.4 Incidence of pregnancy loss and performance of the BPAG assays

An incidence of pregnancy loss of 7.5% (70/928) between 28-35 and 42-49 days of gestation was found. The analysis of factors influencing pregnancy loss is presented in Table 3.13. Only days after AI had significant influence ( $P < 0.05$ ) on pregnancy loss. However, days in milk, lactation number and breed had no significant influence on pregnancy loss (Appendix).

Cows that were examined at 28 days after AI were 11.1, times more likely to have pregnancy loss compared to cows examined at 35 days after AI ( $P = 0.026$ ). Cows that were examined at 30 days after AI were 11.1, times more likely to have pregnancy loss compared to cows examined at 35 days after AI ( $P = 0.021$ ). Cows that were examined at 31 days after AI were 11.0, times more likely to have pregnancy loss compared to cows examined at 35 days after AI ( $P = 0.021$ ). Cows that were examined at 34 days after AI were 11.1, times more likely to have pregnancy loss compared to cows examined at 35 days after AI ( $P = 0.024$ ).

Table 3.13: Logistic regression model showing influence of days after AI on pregnancy loss based on TRUS B

| Variable      | Parameter estimate<br>(B) | P-value | Odds ratio (95% CI)   |
|---------------|---------------------------|---------|-----------------------|
| Days after AI |                           |         |                       |
| 28            | 2.411                     | 0.026   | 11.148 (1.334-93.132) |
| 29            | 1.537                     | 0.158   | 4.649 (0.549-39.339)  |
| 30            | 2.411                     | 0.021   | 11.148 (1.437-86.462) |
| 31            | 2.396                     | 0.021   | 10.979 (1.440-83.714) |
| 32            | 1.746                     | 0.097   | 5.733 (0.728-45.148)  |
| 33            | 1.870                     | 0.081   | 6.491 (0.796-52.909)  |
| 34            | 2.387                     | 0.024   | 10.886 (1.362-86.980) |
| 35            | Referent                  | -       | -                     |

Additional analysis was done to evaluate the serum and milk PAG levels of cows undergoing pregnancy loss (Fig. 3.15 and 3.16). Each day for the days after AI represents a group of cows with the average serum BPAG A levels. The serum BPAG A levels were high from 28 days until 34 days after AI and gradually decreased from 34 days until 49 days after AI (Fig. 3.9). The milk BPAG A levels significantly increased from 29 days until 34 days after AI and gradually decreased from 34 days and rose from 47 days after AI (Fig. 3.10).

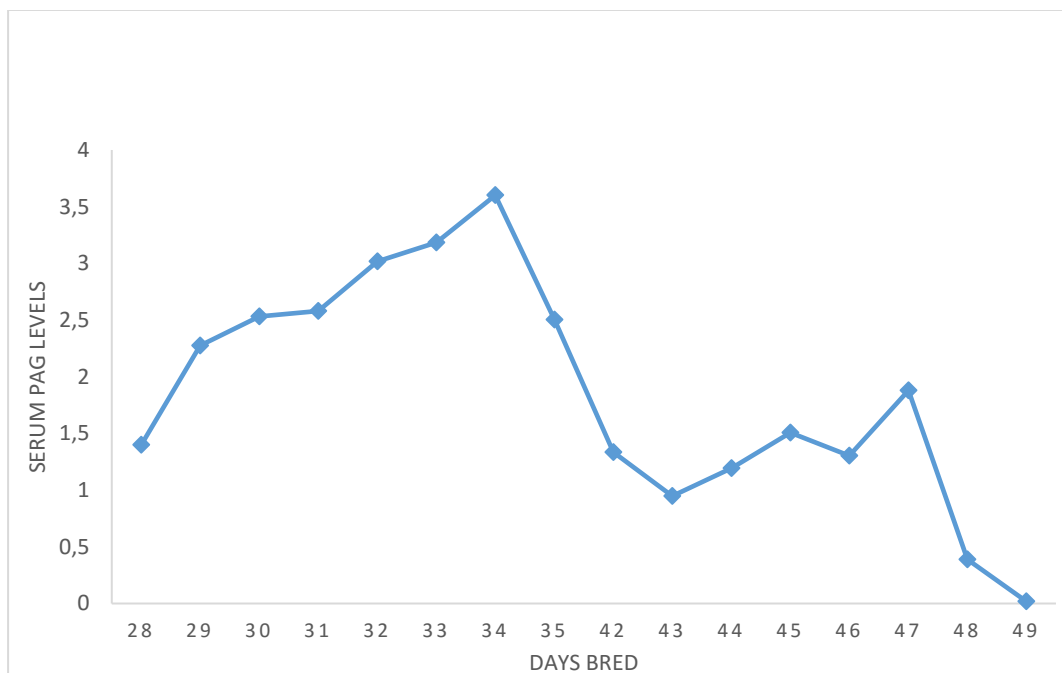


Figure 3.9: Serum PAG A levels of cows experiencing pregnancy loss based on TRUS B.

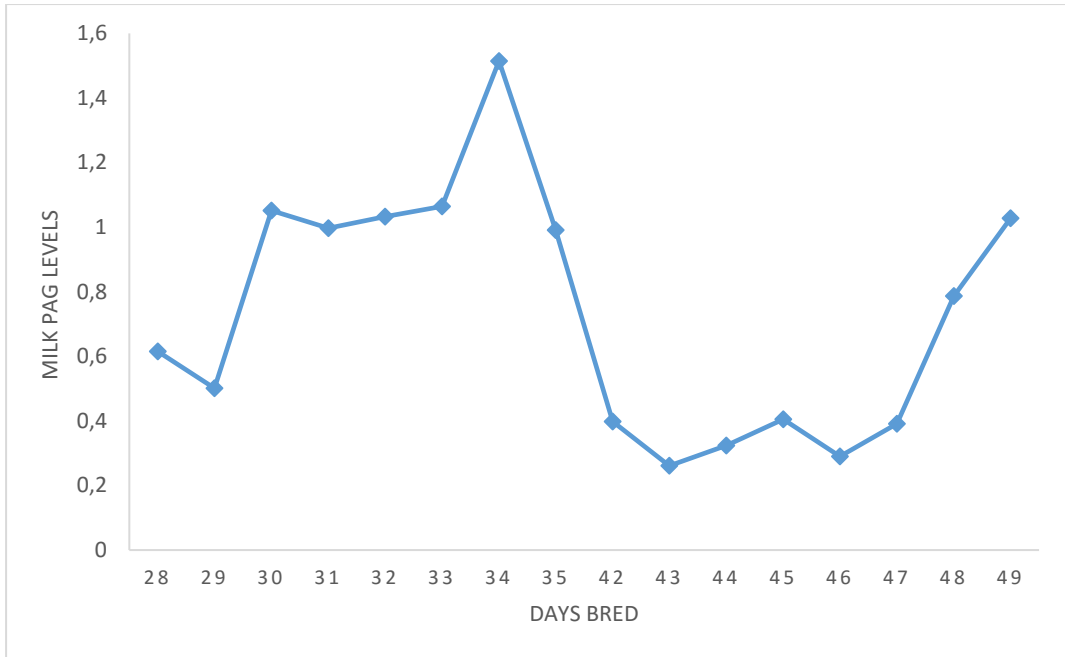


Figure 3.10: Milk PAG A levels of cows experiencing pregnancy loss based on TRUS B.

## CHAPTER FOUR: DISCUSSION

### 4.1 Test performance of the three BPAG A ELISA tests

This study was designed to evaluate the test performance of three commercial PAG ELISAs relative to transrectal ultrasound for doing early pregnancy diagnosis in South African dairy cows.

Blood and milk was collected from days 28 to 35 after AI and concurrently ultrasound examination was done. This first sampling was labelled as 'A'. The BPAG A ELISA were tested and compared with ultrasound results. After comparison, there were discrepancies between the BPAG ELISAs and ultrasound results. Therefore, blood and milk were collected again two weeks later for BPAG A ELISA that disagreed with ultrasound. The ultrasound at second sampling was considered as the reference test between the comparison of PAG ELISA and ultrasound.

Possible causes of discrepancies between the BPAG A ELISA and ultrasound A include the fact that the practitioners were not used to diagnosing pregnancy at such an early stage in most cases (See data capture, Appendix), differentiation of other causes of fluid accumulation in the uterus, and the fact that the study was done by practitioners in commercial herds where it is not possible to perform examinations at a sufficiently slow pace to ensure a very high degree of diagnostic accuracy. After 42 days the accuracy of pregnancy diagnosis by ultrasound is much higher and therefore a suitable reference point for comparison. The impact of this on the results would have been minor if the incidence of pregnancy loss was in line with other studies published elsewhere. The Bayesian method is another analytical method which does not require a reference test and is the subject of on-going work beyond the scope of this dissertation.

In this study, the serum, milk and serum visual BPAG A ELISA estimated sensitivity of 99.8, 99.4 and 99.6%, respectively relative to TRUS B from 928 inseminated dairy cows at 28 to 35 days after AI. These results are similar to the findings of (Silva et al. 2007, Green et al. 2009, LeBlanc 2013) where these investigators found sensitivity of 93.9, 99.6 and 99.2%, respectively, as early as 27 to 29 days after AI relative to transrectal ultrasound. The results of our study are in line with other investigators. This study found few false negatives meaning that the BPAG ELISAs was in close agreement with the practitioners' examination. In practice, cows found to be open at pregnancy examination are often given prostaglandin  $F_{2\alpha}$ , in the case of animals with a false negative diagnosis would cause abortion (Thurmond, Picanso & Jameson 1990, De Vries 2006). However, in research false negatives lead to erroneous data and inconclusive results (Romano, Larson 2010).

On the other hand, the serum, milk and serum visual BPAG A ELISA estimated specificity of 57.3, 55.3 and 55.0%, respectively relative to TRUS B. These results do not agree with the findings of (Silva et al. 2007, Green et al. 2009, LeBlanc 2013) where PAG ELISA estimated

specificity of 95.5, 91.7 and 95.5%, respectively, when compared to transrectal ultrasound. The reason for lower specificity values is due to the number of false positives found in this study.

This study found that the serum, milk and serum visual BPAG A ELISA estimated false positives  $n=171$ , 178 and 179, respectively. One possibility of false positives is because of the occurrence of pregnancy loss of 7.5% (70/928) found in this study. However, this study speculates that the incidence of pregnancy loss is not the only cause of lower specificity. There is possibility of circulating PAGs in the maternal system. According to Whitlock and co-workers, (2008), lower specificity in early gestation compared to late gestation is likely due to higher rates of pregnancy loss in early gestation and slow clearance of circulating BPAGs. The half-life of PAG in the blood stream was found to be 4.3 days during the postpartum period (Green et al. 2005) and 5 to 7 days after induction of embryo mortality in dairy cows at 39 days of pregnancy (Giordano et al. 2012). The BPAG ELISAs can detect PAG levels that decreased after pregnancy loss leading to false positives whereas ultrasound can be used to visualize embryo death and reduce the number of false positives (Silva et al. 2007). It is advisable to trace back cows that lost their pregnancies and it is proven to be an economical approach taking into account multiple factors, including the cost of additional examination (Giordano, Fricke & Cabrera 2013).

Another possibility is the problem of circulating PAGs postpartum from the previous pregnancy. (Silva et al. 2007) suggested that if cows are to be sampled within 70 days postpartum, the PAG assay can still detect circulating PAGs from previous pregnancy which increases the rate of false-positives and thereby reducing the specificity of the assay. None of the cows in the present study were within this time frame after calving.

A warning in this study is that pregnancy loss could have occurred before the time frame (28 to 35 days) of this study. Furthermore, pregnancy loss could have also occurred between the first sampling and two weeks later (42 to 49 days). It is a challenge to know when exactly pregnancy loss occurred. Therefore, the results of lower specificity in this study are likely to be confounded by pregnancy loss either preceding or after sample-collection at 28-35 days after AI. The fact that the BPAG ELISAs were all highly sensitive in identifying pregnant cows but poorly specific in identifying non-pregnant cows compared to transrectal ultrasonography renders them to be moderately satisfactory methods for early pregnancy diagnosis in South African dairy cows.

#### 4.2 Factors influencing sensitivity and specificity of serum, milk and visual BPAG ELISAs

Results of the present study indicate that days after AI and breed had significant influence on specificity of the serum, milk and serum visual BPAG ELISAs. Cows that were examined at 28 and 29 days after AI had higher specificity estimates because of fewer false-positive reaction in the serum, milk and serum visual BPAG ELISAs. The reason for the few false-positives at 28 and 29 days after AI could be that those cows lost fewer pregnancies than those samples on days 30-35. This suggests that testing for pregnancy at 28 and 29 days after AI could be the best period for sampling. The specificity estimates decreased as the days after AI increased.

The specificity was higher for Holstein cows than Jersey and crossbred cows. This finding could mean that Holstein cows could have had lower rates of pregnancy loss than Jersey and crossbred cows. However, further studies are required to examine the BPAG levels of various dairy cow breeds during pregnancy as well as the incidence of pregnancy loss.

This section concludes that days after AI (28 and 29) post breeding and Holstein cow breed indirectly influenced the specificity estimates of all the BPAG ELISAs. The BPAG ELISAs identified open cows fairly at 28 and 29 days after AI and in Holstein cows.

#### 4.3 Incidence of pregnancy loss and performance of the BPAG assays

The incidence of pregnancy loss was not a primary focus of the study however due to low specificity estimates, the issue of pregnancy loss was later investigated.

This study found that days after AI post breeding was the only factor that indirectly influenced pregnancy loss. This study found an incidence of pregnancy loss of 7.5 %. This study found that cows at 28, 30, 31 and 34 days after AI are more likely to experience pregnancy losses. (Silke et al. 2002) reported that about half of the late embryo losses occurs between 28 and 42 days of gestation. Multiple studies have documented pregnancy loss particularly late embryo mortality ranging from 3.2 to 50% (Zobel et al. 2011, Ribeiro et al. 2011, Ribeiro et al. 2012, Ribeiro et al. 2013). The timing of embryo loss between 30 to 60 days of gestation suggests that placental function is compromised because this timing corresponds to the establishment of a functional cotyledonary placenta in cattle taking place, for example between 25 and 40 days as per Pfarrer et al., 2006; Aires et al., 2014) as cited by Pohler et al. (2013).

It has been recommended that pregnancy diagnosis should be performed as early as possible post breeding without having the diagnosis confounded by subsequent pregnancy loss (Studer 1969; Melrose 1979) as cited by (Ricci et al. 2015)

Pregnancy loss was regarded as a confounder which diminishes the benefit of doing early pregnancy diagnosis in two ways. Firstly, there is a high rate of pregnancy loss at the time when most pregnancy diagnoses are done (Santos et al. 2004). Consequently, when pregnancy diagnosis is done earlier, fewer cows identified as non-pregnant are eligible for re-insemination. Secondly, cows initially identified as pregnant earlier endure a high risk of pregnancy loss compared with cows identified as pregnant at later stage of gestation. If not identified, cows with subsequent pregnancy losses reduce the reproductive efficiency by extending the calving interval to conception (Ricci et al. 2015).

This study traced PAG levels at from 28 to 35 days after AI and two weeks later (42 to 49 days after AI). Results show that PAG levels were higher at 28 to 35 days after AI but later decreased at 42 to 49 days after AI. This study found cows with high levels of PAGs that had lost pregnancies. The PAG levels rose from 46 days after AI for the serum PAG ELISA and then later dropped until 49 days after AI. Moreover, milk PAG levels rose from 46 days after AI until 49 days after AI. These findings are similar to a study by (López-Gatius et al. 2007a) where a high risk of pregnancy loss was reported with cows that had high BPAG levels than those with



medium levels. It is difficult to explain why an increase in BPAG levels are associated with pregnancy loss since they are identified as pregnancy markers (Green et al. 2005) and play a role of protecting the embryo against maternal rejection (Wooding, Roberts & Green 2005).

A study by López-Gatius et al. (2007b) hypothesized that cows under heat stress may have increased BPAG levels as a result of the mother failing to respond to embryonic signals. In the same study, the risk of pregnancy loss was 6.8 times greater in cows with high BPAG levels in early pregnancy than those with average BPAG levels (López-Gatius et al. 2007b). This information requires extensive research to draw up conclusions for dairy cows undergoing pregnancy loss with high BPAG levels. Conversely, low circulating PAGs in cows undergoing pregnancy loss may be a good marker for detecting early pregnancy loss (Pohler et al. 2013).

It is challenge to compare pregnancy loss rates by geography, the study population, the study design and collection days of samples at gestation. This study eliminated the effect of farm management (practices, environment), herd and geographical location in relation to how these factors may contribute to pregnancy loss in dairy cows. Therefore, extensive studies are required to evaluate factors that contribute to early pregnancy loss in South African dairy herds.

Briefly, this section concludes that days after AI indirectly influenced pregnancy loss because BPAG levels decreased two weeks later. However, pregnancy loss is speculated to be a confounding factor diminishing pregnancy diagnosis based on BPAG ELISAs when compared with transrectal ultrasonography.

## **CHAPTER FIVE: CONCLUSIONS**

All BPAG ELISAs evaluated were highly sensitive in identifying pregnant cows between 28 and 35 days of pregnancy but poorly specific in identifying cows which were non-pregnant two weeks later. Therefore, the BPAG ELISAs proved to be moderately satisfactory methods for early pregnancy diagnosis in South African dairy cows. Furthermore, this study concludes that days after AI indirectly influenced pregnancy loss because BPAG levels decreased two weeks later. However, pregnancy loss was found to be a confounding factor diminishing pregnancy diagnosis based on BPAG ELISAs when compared with transrectal ultrasonography. These results indicate a limitation of the BPAG ELISAs which overestimated the rate of truly pregnant cows. Therefore, further studies are required to modify this weakness of the assays.

## **CHAPTER SIX: RECOMMENDATIONS**

This study recommends farmers or veterinarians to perform diagnosis with the BPAG ELISAs at a later stage than the period selected for this study due to high rates of pregnancy losses experienced in early gestation or to confirm pregnancy by follow-up examination in cows diagnosed as pregnant using BPAG ELISAs in early pregnancy.

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**APPENDICES**

Appendix A: Data capture sheet bovine pregnancy test trial.

| Sample | Farm | Animal ID | Calving date | Breeding date | Date of exam & sample collection | Result of ultrasound<br>Pregnant (+)/ Open (NIC) | Comment other observations |
|--------|------|-----------|--------------|---------------|----------------------------------|--|----------------------------|
| 1      |      |           |              |               |                                  |  |                            |
| 2      |      |           |              |               |                                  |  |                            |
| 3      |      |           |              |               |                                  |  |                            |
| 4      |      |           |              |               |                                  |  |                            |
| 5      |      |           |              |               |                                  |  |                            |
| 6      |      |           |              |               |                                  |  |                            |
| 7      |      |           |              |               |                                  |  |                            |

Appendix B: Veterinary questionnaire for ultrasound examination

Name of practitioner: \_\_\_\_\_ Date: \_\_\_\_\_

1. Which ultrasound scanner and at what frequency was used?
  - a) Scanner
  - b) Probe
2. Criteria used for pregnancy diagnosis?
  - a) Presence of clear fluid
  - b) Presence of membranes
  - c) Presence of embryo or foetus
  - d) Heart beat
  - e) Other, specify \_\_\_\_\_
3. Restraint during pregnancy examination?
  - a) Milking parlour
  - b) Head gates
  - c) Palpation rail
  - d) Other, specify \_\_\_\_\_
4. How were the results for pregnancy examination recorded?
  - a) Captured on hand-written list
  - b) Captured on computer-generated list
  - c) Other, specify \_\_\_\_\_
5. Number of years' experience using the ultrasound for pregnancy diagnosis?  
\_\_\_\_\_
6. Normal routine for performing pregnancy diagnosis \_\_\_\_\_.

Appendix C

Appendix C1: No effect of days after AI on sensitivity of the serum A BPAG A ELISA.

|                  | B           | S.E      | Wald | df | Sig.  | Exp   | 95% C.I for EXP<br>(B) |       |
|------------------|-------------|----------|------|----|-------|-------|------------------------|-------|
|                  |             |          |      |    |       |       | Lower                  | Upper |
| Step 1           |             |          | .000 | 7  | 1.000 |       |                        |       |
| Days after AI    |             |          |      |    |       |       |                        |       |
| Days after AI 28 | .000        | 9223.690 | .000 | 1  | 1.000 | 1.000 | .000                   | .     |
| Days after AI 29 | .000        | 7254.152 | .000 | 1  | 1.000 | 1.000 | .000                   | .     |
| Days after AI 30 | .000        | 7201.237 | .000 | 1  | 1.000 | 1.000 | .000                   | .     |
| Days after AI 31 | .000        | 6915.922 | .000 | 1  | 1.000 | 1.000 | .000                   | .     |
| Days after AI 32 | .000        | 6506.845 | .000 | 1  | 1.000 | 1.000 | .000                   | .     |
| Days after AI 33 | .000        | 7080.543 | .000 | 1  | 1.000 | 1.000 | .000                   | .     |
| Days after AI 34 | -<br>17.271 | 5232.679 | .000 | 1  | .997  | .000  | .000                   | .     |



Appendix C2: No effect of breed on sensitivity of the serum A BPAG AELSA

|           | B           | S.E      | Wald | Df | Sig.  | Exp   | 95% C.I for EXP<br>(B) |       |
|-----------|-------------|----------|------|----|-------|-------|------------------------|-------|
|           |             |          |      |    |       |       | Lower                  | Upper |
| Step 1    |             |          | .000 | 2  | 1.000 |       |                        |       |
| Breed_cat |             |          |      |    |       |       |                        |       |
| Holstein  | -<br>15.156 | 7338.199 | .000 | 1  | .998  | .000  | .000                   | .     |
| Jersey    | .000        | 9152.342 | .000 | 1  | 1.000 | 1.000 | .000                   | .     |

Appendix C3: No effect of lactation number on sensitivity of the serum BPAG A ELISA

|             | B      | S.E       | Wald | Df | Sig.  | Exp   | 95%C.I for EXP (B) |       |
|-------------|--------|-----------|------|----|-------|-------|--------------------|-------|
|             |        |           |      |    |       |       | Lower              | Upper |
| Step 1      |        |           | .000 | 5  | 1.000 |       |                    |       |
| Lact_num    |        |           |      |    |       |       |                    |       |
| Lactation 1 | .000   | 16751.784 | .000 | 1  | 1.000 | 1.000 | .000               | .     |
| Lactation 2 | -      | 16408.708 | .000 | 1  | .999  | .000  | .000               | .     |
|             | 16.530 |           |      |    |       |       |                    |       |
| Lactation 3 | .000   | 17097.483 | .000 | 1  | 1.000 | 1.000 | .000               | .     |
| Lactation 4 | .000   | 17625.780 | .000 | 1  | 1.000 | 1.000 | .000               | .     |
| Lactation 5 | .000   | 17974.840 | .000 | 1  | 1.000 | 1.000 | .000               | .     |

Appendix C4: No effect of days in milk (DIM) on sensitivity of the serum BPAG A ELISA

|             |   | B      | S.E       | Wald | df | Sig.  | Exp   | 95%C.I for EXP<br>(B) |       |
|-------------|---|--------|-----------|------|----|-------|-------|-----------------------|-------|
|             |   |        |           |      |    |       |       | Lower                 | Upper |
| Step 1 DIM  |   |        |           | .000 | 3  | 1.000 |       |                       |       |
| DIM 42-194  | - |        | 40192.955 | .000 | 1  | 1.000 | .000  | .000                  | .     |
|             |   | 15.646 |           |      |    |       |       |                       |       |
| DIM 195-348 |   | .000   | 40352.136 | .000 | 1  | 1.000 | 1.000 | .000                  | .     |
| DIM 349-501 |   | .000   | 41834.155 | .000 | 1  | 1.000 | 1.000 | .000                  | .     |

Appendix C5: No effect of days after AI on sensitivity of the milk BPAG A ELISA

|               | B      | S.E      | Wald | df | Sig.  | Exp          | 95%C.I for EXP (B) |        |
|---------------|--------|----------|------|----|-------|--------------|--------------------|--------|
|               |        |          |      |    |       |              | Lower              | Upper  |
| Step 1        |        |          | .020 | 7  | 1.000 |              |                    |        |
| Days after AI |        |          |      |    |       |              |                    |        |
| 28            | 17.178 | 7882.490 | .000 | 1  | .998  | 28847765.051 | .000               | .      |
| 29            | .035   | 1.427    | .001 | 1  | .980  | 1.036        | .063               | 16.964 |
| 30            | 17.178 | 4985.324 | .000 | 1  | .997  | 28847765.051 | .000               | .      |
| 31            | 17.178 | 4550.958 | .000 | 1  | .997  | 28847765.051 | .000               | .      |
| 32            | 17.178 | 3922.432 | .000 | 1  | .997  | 28847765.051 | .000               | .      |
| 33            | 17.178 | 4803.979 | .000 | 1  | .997  | 28847765.051 | .000               | .      |
| 35            |        |          |      |    |       |              |                    |        |

Appendix C6: No effect of breed on the sensitivity of the milk BPAG A ELISA.

|           | B      | S.E      | Wald | df | Sig.  | Exp   | 95%C.I for EXP (B) |       |
|-----------|--------|----------|------|----|-------|-------|--------------------|-------|
|           |        |          |      |    |       |       | Lower              | Upper |
| Step 1    |        |          | .000 | 2  | 1.000 |       |                    |       |
| breed_cat |        |          |      |    |       |       |                    |       |
| Holstein  | -      | 7218.871 | .000 | 1  | .998  | .000  | .000               | .     |
|           | 16.259 |          |      |    |       |       |                    |       |
| Jersey    | .000   | 9056.948 | .000 | 1  | 1.000 | 1.000 | .000               | .     |

Appendix C7: No effect of lactation number on sensitivity of the milk BPAG A ELISA.

|             | B       | S.E       | Wald | df | Sig.  | Exp   | 95%C.I for EXP<br>(B) |       |
|-------------|---------|-----------|------|----|-------|-------|-----------------------|-------|
|             |         |           |      |    |       |       | Lower                 | Upper |
| Step 1      |         |           | .619 | 5  | .987  |       |                       |       |
| Lact_num    |         |           |      |    |       |       |                       |       |
| Lactation 1 | -16.254 | 16408.763 | .000 | 1  | .999  | .000  | .000                  | .     |
| Lactation 2 | -17.223 | 16408.763 | .000 | 1  | .999  | .000  | .000                  | .     |
| Lactation 3 | .000    | 17097.535 | .000 | 1  | 1.000 | 1.000 | .000                  | .     |
| Lactation 4 | .000    | 17625.831 | .000 | 1  | 1.000 | 1.000 | .000                  | .     |
| Lactation 5 | .000    | 17974.890 | .000 | 1  | 1.000 | 1.000 | .000                  | .     |

Appendix C8: No effect of DIM on sensitivity of the milk BPAG A ELISA.

|             |       | B      | S.E       | Wald  | df | Sig.  | Exp   | 95%C.I for EXP<br>(B) |       |
|-------------|-------|--------|-----------|-------|----|-------|-------|-----------------------|-------|
|             |       |        |           |       |    |       |       | Lower                 | Upper |
| Step 1 DIM  |       |        |           | 0.001 | 3  | 1.000 |       |                       |       |
| DIM 42-194  | -     |        | 40192.710 | 0.000 | 1  | 1.000 | 0.000 | 0.000                 | .     |
|             |       | 16.343 |           |       |    |       |       |                       |       |
| DIM 195-348 | -     |        | 40192.710 | 0.000 | 1  | 1.000 | 0.000 | 0.000                 | .     |
|             |       | 16.375 |           |       |    |       |       |                       |       |
| DIM 349-501 | 0.000 |        | 41833.920 | 0.000 | 1  | 1.000 | 1.000 | 0.000                 | .     |

Appendix C9: No effect of days after AI on the sensitivity of the serum visual BPAG A ELISA.

|               | B       | S.E      | Wald | df | Sig.  | Exp   | 95%C.I for EXP (B) |       |
|---------------|---------|----------|------|----|-------|-------|--------------------|-------|
|               |         |          |      |    |       |       | Lower              | Upper |
| Step 1        |         |          | .233 | 7  | 1.000 |       |                    |       |
| Days after AI |         |          |      |    |       |       |                    |       |
| 28            | -18.025 | 5323.689 | .000 | 1  | .997  | .000  | .000               | .     |
| 29            | .000    | 7464.757 | .000 | 1  | 1.000 | 1.000 | .000               | .     |
| 30            | .000    | 7293.498 | .000 | 1  | 1.000 | 1.000 | .000               | .     |
| 31            | .000    | 7022.952 | .000 | 1  | 1.000 | 1.000 | .000               | .     |
| 32            | .000    | 6612.650 | .000 | 1  | 1.000 | 1.000 | .000               | .     |
| 33            | .000    | 7170.765 | .000 | 1  | 1.000 | 1.000 | .000               | .     |
| 34            | -17.332 | 5323.689 | .000 | 1  | .997  | .000  | .000               | .     |



Appendix C10: No effect of breed on sensitivity of the serum visual BPAG A ELISA.

|           | B           | S.E      | Wald | d.f | Sig.  | Exp   | 95%C.I for EXP<br>(B) |       |
|-----------|-------------|----------|------|-----|-------|-------|-----------------------|-------|
|           |             |          |      |     |       |       | Lower                 | Upper |
| Step 1    |             |          | .000 | 2   | 1.000 |       |                       |       |
| Breed_cat |             |          |      |     |       |       |                       |       |
| Holstein  | -<br>15.851 | 7338.199 | .000 | 1   | .998  | .000  | .000                  | .     |
| Jersey    | .000        | 9183.127 | .000 | 1   | 1.000 | 1.000 | .000                  | .     |

Appendix C11: No effect of lactation number on sensitivity of the serum visual BPAG A ELISA.

|             | B      | S.E       | Wald | df | Sig.  | Exp   | 95%C.I for EXP (B) |       |
|-------------|--------|-----------|------|----|-------|-------|--------------------|-------|
|             |        |           |      |    |       |       | Lower              | Upper |
| Step 1      |        |           | .000 | 5  | 1.000 |       |                    |       |
| Lact_num    |        |           |      |    |       |       |                    |       |
| Lactation 1 | .000   | 16751.784 | .000 | 1  | 1.000 | 1.000 | .000               | .     |
| Lactation 2 | -      | 16408.708 | .000 | 1  | .999  | .000  | .000               | .     |
|             | 17.233 |           |      |    |       |       |                    |       |
| Lactation 3 | .000   | 17097.483 | .000 | 1  | 1.000 | 1.000 | .000               | .     |
| Lactation 4 | .000   | 17625.780 | .000 | 1  | 1.000 | 1.000 | .000               | .     |
| Lactation 5 | .000   | 17974.840 | .000 | 1  | 1.000 | 1.000 | .000               | .     |

Appendix C12: No effect of DIM on sensitivity of the serum visual BPAG A ELISA.

|             | B      | S.E       | Wald  | df | Sig.  | Exp   | 95%C.I for EXP<br>(B) |       |
|-------------|--------|-----------|-------|----|-------|-------|-----------------------|-------|
|             |        |           |       |    |       |       | Lower                 | Upper |
| Step 1 DIM  |        |           | 4.752 | 3  | .191  |       |                       |       |
| DIM 42-194  | -      | 40192.923 | .000  | 1  | 1.000 | .000  | .000                  | .     |
|             | 15.650 |           |       |    |       |       |                       |       |
| DIM 195-348 | .000   | 40352.103 | .000  | 1  | 1.000 | 1.000 | .000                  | .     |
| DIM 349-501 | -      | 40192.923 | .000  | 1  | 1.000 | .000  | .000                  | .     |
|             | 18.805 |           |       |    |       |       |                       |       |

Appendix C13: No effect of lactation number on specificity of serum BPAG A ELISA.

|             | B       | S.E           | Wald | df | Sig.  | Exp  | 95%C.I for EXP<br>(B) |       |
|-------------|---------|---------------|------|----|-------|------|-----------------------|-------|
|             |         |               |      |    |       |      | Lower                 | Upper |
| Step 1      |         |               | 2.54 | 7  | .923  |      |                       |       |
| Lact_num    |         |               | 9    |    |       |      |                       |       |
| Lactation 1 | -21.896 | 40196.7<br>40 | .000 | 1  | 1.000 | .000 | .000                  | .     |
| Lactation 2 | -21.804 | 40196.7<br>40 | .000 | 1  | 1.000 | .000 | .000                  | .     |
| Lactation 3 | -21.791 | 40196.7<br>40 | .000 | 1  | 1.000 | .000 | .000                  | .     |
| Lactation 4 | -21.385 | 40196.7<br>40 | .000 | 1  | 1.000 | .000 | .000                  | .     |
| Lactation 5 | -21.714 | 40196.7<br>40 | .000 | 1  | 1.000 | .000 | .000                  | .     |
| Lactation 6 | -22.302 | 40196.7<br>40 | .000 | 1  | 1.000 | .000 | .000                  | .     |
| Lactation 7 | -21.203 | 40196.7<br>40 | .000 | 1  | 1.000 | .000 | .000                  | .     |

Appendix C14: No effect of DIM on specificity of serum BPAG A ELISA.

|                 | B       | S.E       | Wald  | df | Sig.  | Exp  | 95%C.I for EXP (B) |       |
|-----------------|---------|-----------|-------|----|-------|------|--------------------|-------|
|                 |         |           |       |    |       |      | Lower              | Upper |
| Step 1<br>DIM   |         |           | 4.292 | 3  | .232  |      |                    |       |
| DIM 42-<br>194  | -21.612 | 40221.453 | .000  | 1  | 1.000 | .000 | .000               | .     |
| DIM 195-<br>348 | -21.795 | 40221.453 | .000  | 1  | 1.000 | .000 | .000               | .     |
| DIM 349-<br>501 | -23.149 | 40221.453 | .000  | 1  | 1.000 | .000 | .000               | .     |

Appendix C15: No effect of lactation number on specificity of milk BPAG A ELISA.

|             | B      | S.E       | Wald  | df | Sig.  | Exp  | 95%C.I for EXP<br>(B) |       |
|-------------|--------|-----------|-------|----|-------|------|-----------------------|-------|
|             |        |           |       |    |       |      | Lower                 | Upper |
| Step 1      |        |           | 1.769 | 7  | .972  |      |                       |       |
| Lact_num    |        |           |       |    |       |      |                       |       |
| Lactation 1 | -      | 40197.663 | .000  | 1  | 1.000 | .000 | .000                  | .     |
|             | 21.726 |           |       |    |       |      |                       |       |
| Lactation 2 | -      | 40197.663 | .000  | 1  | 1.000 | .000 | .000                  | .     |
|             | 21.608 |           |       |    |       |      |                       |       |
| Lactation 3 | -      | 40197.663 | .000  | 1  | 1.000 | .000 | .000                  | .     |
|             | 21.854 |           |       |    |       |      |                       |       |
| Lactation 4 | -      | 40197.663 | .000  | 1  | 1.000 | .000 | .000                  | .     |
|             | 21.571 |           |       |    |       |      |                       |       |
| Lactation 5 | -      | 40197.663 | .000  | 1  | 1.000 | .000 | .000                  | .     |
|             | 21.203 |           |       |    |       |      |                       |       |
| Lactation 6 | -      | 40197.663 | .000  | 1  | 1.000 | .000 | .000                  | .     |
|             | 21.714 |           |       |    |       |      |                       |       |
| Lactation 7 | -      | 40197.663 | .000  | 1  | 1.000 | .000 | .000                  | .     |
|             | 21.203 |           |       |    |       |      |                       |       |

Appendix C16: No effect of DIM on specificity of milk BPAG A ELISA.

|             | B | S.E    | Wald      | df   | Sig. | Exp   | 95%C.I for EXP<br>(B) |       |
|-------------|---|--------|-----------|------|------|-------|-----------------------|-------|
|             |   |        |           |      |      |       | Lower                 | Upper |
| Step 1 DIM  |   |        | 4.355     | 3    | .226 |       |                       |       |
| DIM 42-194  | - | 21.552 | 40209.899 | .000 | 1    | 1.000 | .000                  | .     |
| DIM 195-348 | - | 21.620 | 40209.899 | .000 | 1    | 1.000 | .000                  | .     |
| DIM 349-501 | - | 23.149 | 40209.899 | .000 | 1    | 1.000 | .000                  | .     |

Appendix C17: No effect of breed on specificity of serum visual BPAG A ELISA.

|           | B       | S.E       | Wald   | df | Sig.  | Exp   | 95%C.I for EXP<br>(B) |       |
|-----------|---------|-----------|--------|----|-------|-------|-----------------------|-------|
|           |         |           |        |    |       |       | Lower                 | Upper |
| Step 1    |         |           | 19.357 | 2  | 0.000 |       |                       |       |
| Breed_cat |         |           |        |    |       |       |                       |       |
| Holstein  | -21.686 | 10048.291 | 0.000  | 1  | 0.998 | 0.000 | 0.000                 | -     |
| Jersey    | -18.969 | 10048.291 | 0.000  | 1  | 0.998 | 0.000 | 0.000                 | -     |



Appendix C18: No effect of lactation number on specificity of serum visual BPAG A ELISA.

|             | B       | S.E       | Wald  | df | Sig.  | Exp   | 95%C.I for EXP (B) |       |
|-------------|---------|-----------|-------|----|-------|-------|--------------------|-------|
|             |         |           |       |    |       |       | Lower              | Upper |
| Step 1      |         |           | 1.869 | 7  | 0.967 |       |                    |       |
| Lact_num    |         |           |       |    |       |       |                    |       |
| Lactation 1 | -21.776 | 40176.335 | 0.000 | 1  | 1.000 | 0.000 | 0.000              | -     |
| Lactation 2 | -21.669 | 40176.335 | 0.000 | 1  | 1.000 | 0.000 | 0.000              | -     |
| Lactation 3 | -21.608 | 40176.335 | 0.000 | 1  | 1.000 | 0.000 | 0.000              | -     |
| Lactation 4 | -21.385 | 40176.335 | 0.000 | 1  | 1.000 | 0.000 | 0.000              | -     |
| Lactation 5 | -21.713 | 40176.335 | 0.000 | 1  | 1.000 | 0.000 | 0.000              | -     |
| Lactation 6 | -22.301 | 40176.335 | 0.000 | 1  | 1.000 | 0.000 | 0.000              | -     |
| Lactation 7 | -21.203 | 40176.335 | 0.000 | 1  | 1.000 | 0.000 | 0.000              | -     |

Appendix C19: No effect of DIM on specificity of serum visual BPAG A ELISA.

|             | B       | S.E       | Wald  | df | Sig.  | Exp   | 95%C.I for EXP (B) |       |
|-------------|---------|-----------|-------|----|-------|-------|--------------------|-------|
|             |         |           |       |    |       |       | Lower              | Upper |
| Step 1 DIM  |         |           | 2.984 | 3  | 0.394 |       |                    |       |
| DIM 42-194  | -21.557 | 40176.450 | 0.000 | 1  | 1.000 | 0.000 | 0.000              | -     |
| DIM 195-348 | -21.677 | 40176.450 | 0.000 | 1  | 1.000 | 0.000 | 0.000              | -     |
| DIM 349-501 | -22.669 | 40176.450 | 0.000 | 1  | 1.000 | 0.000 | 0.000              | -     |

APPENDIX D

Appendix D1: No effect of lactation number on pregnancy loss

| Variable         | $\beta$ | S.E       | Wald  | Df | Sig.  | Exp ( $\beta$ ) | 95% C.I     |             |
|------------------|---------|-----------|-------|----|-------|-----------------|-------------|-------------|
|                  |         |           |       |    |       |                 | Lower Bound | Upper Bound |
| Lactation number |         |           | 2.295 | 7  | .942  |                 |             |             |
| Lactation 1      | 19.859  | 40197.894 | .000  | 1  | 1.000 | 421444221.188   | .000        | .           |
| Lactation 2      | 19.737  | 40197.894 | .000  | 1  | 1.000 | 372816041.820   | .000        | .           |
| Lactation 3      | 19.627  | 40197.894 | .000  | 1  | 1.000 | 334248865.080   | .000        | .           |
| Lactation 4      | 20.009  | 40197.894 | .000  | 1  | 1.000 | 489556418.552   | .000        | .           |
| Lactation 5      | 18.495  | 40197.894 | .000  | 1  | 1.000 | 107702412.081   | .000        | .           |
| Lactation 6      | .000    | 42635.725 | .000  | 1  | 1.000 | 1.000           | .000        | .           |
| Lactation 7      | .000    | 49230.154 | .000  | 1  | 1.000 | 1.000           | .000        | .           |

Appendix D2: No effect of DIM on pregnancy loss

| Variable     | $\beta$ | S.E       | Wald  | Df | Sig.  | Exp ( $\beta$ ) | 95% C.I     |             |
|--------------|---------|-----------|-------|----|-------|-----------------|-------------|-------------|
|              |         |           |       |    |       |                 | Lower Bound | Upper Bound |
| Days in milk |         |           | 3.123 | 3  | .373  |                 |             |             |
| DIM 42-194   | 19.575  | 40191.434 | .000  | 1  | 1.000 | 317183765.445   | .000        | .           |
| DIM195-348   | 18.959  | 40191.434 | .000  | 1  | 1.000 | 171332879.643   | .000        | .           |
| DIM 349-501  | 20.104  | 40191.434 | .000  | 1  | 1.000 | 538474764.592   | .000        | .           |