Pregnancy-associated glycoprotein (PAG) profiles in cows and goats and attempts to measure PAG in milk

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Dedication

To the Soul of my late father

My beloved mother

My dear sister Dima and her husband Reda

and their daughter Rafif

My precious sister Lama

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List of Abbreviations

°C	Celsius
AI	Artificial insemination
cDNA	Complementary deoxyribonucleic acid
e.g.	Abbreviation of latin *exempli gratia* (for example)
ELISA	Enzyme-Linked Immunosorbent Assay
i.e.	Abbreviation of latin *id est* (that is; in other words)
IgG	Immunoglobulin G
kDa	Kilo Dalton
Kg	Kilogram
KHz	Kilohertz
mg	Milligram
mL	Milliliter
ng	Nanogramm
P	Probability
p.p.	Postpartum
PAG	Pregnancy-associated glycoprotein
RR	Recovery rate
SD	Standard deviation
SEM	Standard error mean
UHT	Ultra high temperature treatment
W	Watt
vs.	Versus

Chapter 1

Review of relevant literature

1.1 The Pregnancy-Associated Glycoproteins (PAGs)

Butler and his coworkers have detected and partially characterized in 1982 two new proteins from bovine embryonic membranes. The first was recognized later as alpha 1-fetoprotein, while the second protein was called pregnancy-specific protein-B (PSPB). It has a molecular weight of 43000 - 53000 Da, and an isoelectric point of 4.0-4.4. Beckers et al. (1988) have also isolated similar pregnancy specific protein and established a radioimmunoassay (RIA) to detect this protein in the maternal blood. This RIA can be used as a method for detecting pregnancy at an early stage. According to Lynch et al. (1992) the PSPB has showed high identity with bPAG-1. Zoli et al. (1991), using the method of Butler et al. (1982), isolated many variants of acidic glycoproteins which appear to be associated with pregnancy. They called this group of glycoproteins "pregnancy-associated glycoproteins" or "PAGs".

PAGs generally belong to the "aspartate proteases" family like some enzymes such as Pepsin, cathepsin D and others. The enzymatic activity of the PAGs seems to be restricted or inactivated by mutations in their catalytic center (Green et al., 1998). Different PAG variants have been detected through cDNA screening or extraction from placental tissues of various animal species. Such as cattle (bPAG: Zoli et al., 1991), sheep (ovPAG: Zoli et al., 1990), horse (Green et al., 1994, 1999), pig (pPAG: Szafranska et al., 1995, 2001a, 2001b, 2002), cat (Gan et al., 1997), camlides (Majewska et al., 2009 and 2011) and goat (caPAG: Garbayo et al., 1998).

Using the reverse transcriptase-PCR (RT-PCR) technique, 22 PAG cDNA transcripts have been screened in bovine placental tissue (Green et al., 2000) as early as day 18 after artificial insemination (AI) (Garbayo et al ., 2008). These PAGs have been separated into 2 groups: an ancient group secreted by the trophoblast mono- and binucleate cells and designated as PAG-II, and more recent group restricted to the trophoblast binucleate cells only (Wooding et al., 2005) and designated as PAG-I. According to placental tissue RNA libraries screening done

by (Garbayo et al., 1999) in goats, 11 transcripts has been found with two members belonging to the ancient PAG group and the others to the more recent group. Only 3 PAG were successfully extracted by the same research group (Garbayo et al., 1998).

The trophoblast binucleate cells are considered as a unique feature in the placentation of ruminants. It resulted from the nuclear division of the mononucleate cells of the trophectoderm without a subsequent cell division (Wooding et al., 1983). It will represent about 20% of the cell population of the trophectoderm from day 20 of pregnancy until parturition.

This increase in the binucleate cells population is coming along with maturation and subsequent migration of the fully granulated binucleate cells to the feto-maternal interface through the apical tight junctions of the trophectoderm (Wooding et al., 1983). The migration coincides with the fusion of the binucleate cells with the uterine epithelial cells. At implantation in cow, there is a transient formation of syncytium by binucleate cells fusing with uterine epithelial cells but only a partial loss of the uterine epithelium. This bovine syncytium is then eliminated by displacement by continuing division of the remaining uterine epithelial cells (King et al., 1979; Wathes and Wooding, 1980), subsequently, during the remainder of the pregnancy in cow.

The “migrated” binucleate cell which fuses with an individual cell derived from the original uterine epithelium to form a transient trinucleate cell which dies after the granules from the original binucleate cell have been released by the means of exocytosis (Wathes and Wooding, 1980; Wooding and Beckers, 1987). Thereafter, the trinucleate cells will be resorbed by the trophectoderm after the exocytosis (Green et al., 1998; Wooding, 1992). Similar mechanism of the migration and fusion of the binucleate cells are noticed in goats, with the exception of formation syncytia with more than 3 nuclea in the case of goats, the PAG will reach the peripheral blood in the same way as in cattle (Wango et al., 1990a and 1990b; Wooding et al., 1992).

The binucleate cells play an important endocrinological role during pregnancy. They are the source of some essential products such as placental lactogen (Currie et al., 1990; Wooding et al., 1992) and pregnancy-associated glycoproteins (Green et al., 1998) in addition to Progesterone and 5 β-pregnanediol production as have been reported in sheep and goats (Wango et al., 1991), respectively. Products from the binucleate cell granules reach maternal tissue and subsequently maternal blood through the route described above.

1.1.1 Proposed physiological roles for the PAGs

It had been hypothesized that PAGs can have local immunosuppressive properties, in the maintenance of the histoincompatible feto-maternal unit (Dosogne et al., 2000; Wooding et al., 2005). It had been suggested, that the phylogenetically more ancient PAG, which are mainly expressed at the microvillar junctions (the feto-maternal interface), can be involved in the establishment of an immunological barrier to protect the trophoblast from the maternal immune system. The members of the newer PAG group are supposed to modulate the maternal immune system depending on the fact that they are restricted to the binucleate cells, which are located in the maternal villi of the placentomes (Wooding et al., 2005).

Del Vecchio et al. (1990) have mentioned that the PSPB/PAG molecules induce the secretion of the PGF2α and PGE2 in the endometrial cell explants, when the last have been treated with PSPB/PAG. Del Vecchio et al. (1996) found that PSPB induces the secretion of PGF2α and PGE2 has and also increased the progesterone secretion by mixed large and small bovine luteal cells from days 17-18 of the pregnancy. Austin et al. (1999) have noticed that PSPB/PAG also increases an alpha-chemokine (granulocyte chemotactic protein-2) secretion, which as suggested to play some role in mediating adhesion, inflammation and angiogenesis associated with the implantation of the embryo. Despite the different studies and speculation about their functions, the real function of the PAGs is still unknown.

1.1.2 PAG concentrations throughout pregnancy in cattle, goats and sheep

The concentrations of PAG throughout pregnancy has been studied thoroughly in the last 20 years, one of the first reports about that is the work of Zoli et al. (1992). In this study, in which a homologous radioimmunoassay (RIA) with polyclonal PAG antibody has been used, the PAG concentration increased continuously from day 20 of pregnancy until day 240 followed by a dramatic increase in the last ten days of pregnancy with maximum concentrations between day 5 and day 1 prepartum. Throughout the postpartum phase, the concentration of PAG decreased steadily and became undetectable after 100 days postpartum.

In another study (Green et al., 2005), a homologous ELISA using different and monoclonal PAG antibodies has been developed. This study reported that the PAG immune reactivity rose rapidly between days 24 and 28. The average concentration of PAG rose to 12.3 ± 4.08 ng/mL in week 5 and then declined until week 8 before rising steadily again. A few weeks prior to parturition the concentration of PAG in maternal serum raised more strongly, peaking during the last week of pregnancy. The PAG concentration decreased gradually after parturition and by 8 weeks post-partum, PAGs were undetectable in 95% of the studied animals.

In zebu cattle (*bos indicus*) the average PAG concentration increased progressively from week 8 to week 35 of gestation followed by a strong increase in the last week of gestation. After delivery, plasma PAG concentrations declined significantly until Week 2 postpartum. Afterwards, PAG concentrations decreased slowly reaching the lowest levels at Week 10 postpartum (Sousa et al., 2003).

In goats the PAG concentration shows a different profile with a significant first increase between day 21 and day 28 and maximum levels between the 5^{th} and 8^{th} week of pregnancy. Thereafter, PAG levels decreased slowly until parturition (Chentouf et al., 2007; Gonzalez et al., 2000) reaching basal levels in the 4^{th} week postpartum (Sousa et al, 1999).

In sheep the plasma PAG profiles are characterized by an initial increase between the 3^{rd} and 4^{th} week, followed by further gradual rise up to the 9^{th} week of pregnancy. Between the 9^{th}

and the 19th week the level remained constant, thereafter a drastic surge occurs, reaching a peak at parturition (Ledezma-Torres et al., 2006).

1.1.3 Factors influencing PAG concentrations throughout pregnancy

In the last 20 years different studies have been done to explore factors that may affect the PAG concentration throughout pregnancy in ruminants, especially in cattle. Besides the rise of the PAG concentration during the course of pregnancy, as noticed by Green et al. (2005), Patel et al. (1997) and Zoli et al. (1992), other influencing factors have been identified. The fetal number could has a positive effect on the PSPB concentration as has been reported by Dobson et al. (1993) from day 60 of pregnancy onwards and for PSP60 as have been noticed by Patel et al. (1995) as early as day 30 of pregnancy and the differences were significant after day 50 of the pregnancy. Similar results have been noticed by Patel et al. (1997) concerning the effect of fetal number on the PAG concentration in cattle. Studies on sheep and goats, in which twining is more frequent, have confirmed the effect of the increased number of fetuses carried by the mother on the PAG concentration throughout pregnancy (Batalha et al., 2001; Ranilla et al., 1997; Sousa et al., 1999; Vandaele et al., 2005). Fetal gender has been reported to have an influence on the PAG levels in cows during different stages of pregnancy in cattle according to different studies. Zoli et al. (1992) have shown that the Holstein cows and heifers carrying male fetuses have higher PAG concentration in comparison with the female fetuses, whereas, in the Hereford cows carrying Holstein fetuses, the cows with female fetuses have higher PAG levels. According to (Ranilla et al., 1994) ewes with male fetuses had higher ovPAG level compared with their counterparts with female fetuses; on the other hand, Wallace et al. (1997) have reported no significant differences in the PSPB levels between male and female singleton fetuses. Lopez-Gatius et al. (2007b) also reported no significant effect of the fetal sex on the PAG concentration, which was in consistency with the results reported by Serrano et al. (2009).

There is also some indication that the use of in vitro produced embryos has an effect on the detectable PAG concentrations. In different studies a significant difference in the PAG concentrations were observed after transfer of IVF or cloned embryos (Breukelman et al., 2005a; Chavatte-Palmer et al., 2006; Vasques et al., 1995).

There are some signs that some reproductive hormones could interact with the PAG secretion in placental tissue. Ayad et al. (2007) observed a positive correlation between progesterone and PAG concentration levels in dairy cows in the first pregnancy trimester. In contrast; Lopez-Gatius et al. (2007a) reported no significant effect of progesterone levels on the PAG concentration. Also estradiol 17-β is proposed to exert some influence on the PSPB (Bridges et al., 1999).

Lopez-Gatius et al. (2007b) showed that early fetal lose can result in an abnormally high or low PAG concentration at day 35 of pregnancy. This group has noticed also that the milk production levels of high producing cows can negatively affect their PAG levels. This may results from the higher metabolic rate in those animals which can result in faster catabolism of the PAG (Lopez-Gatius et al., 2007a). An interaction between day of pregnancy and breed of sire in their effect on the PAG concentration throughout pregnancy have been noticed by Serrano et al. (2009) similar observation has been reported about the effect of fetus sire on the PAG level in pregnant cows (Lopez-Gatius et al., 2007a).

The effect of different breeds on the PAG concentration in different ruminant species was reported in various studies. In sheep, Ranilla et al. (1994) have noticed different PAG profile starting after week 18 of pregnancy of the Churra and Merino sheep. Whereas, Ledezma-Torres et al. (2006) reported no significant differences in the PAG profiles between Blackheaded German Mutton sheep, Rhoen sheep and crossbread Blackheaded German Mutton x Dorper sheep. Sousa et al. (1999) have shown different PAG profiles in two different goat breeds from north-east Brazil. In Moxoto breed the PAG level increased and reached a peak at the 7^{th} week of pregnancy, whereas there was a second elevation in the PAG

level in the Caninde breed between the 17th and 19th weeks of pregnancy. In the case of cattle, there are reports of Mialon et al. (1993) and Lobago et al. (2009) showing a significant effect of the breed of the dam on the PSPB and PAG levels, respectively.

1.1.4 The application of the PAGs in diagnosing and monitoring pregnancy

PAG determination in maternal blood has served as a useful tool for pregnancy diagnosis in ruminants in the last years. Different PAG isoforms are detectable in the peripheral blood as early as the 4th in goats and cattle using different measurement techniques such as radioimmunoassay "RIA" (Sousa et al., 1999; Zoli et al.,1992) and enzyme-linked immunosorbent assays "ELISA" (Friedrich and Holtz, 2010; Green et al., 2005).

Different homologous and heterologous immunoassays have been established for determining PAG concentration in cattle (Zoli et al., 1992; Green et al., 2005; Friedrich and Holtz, 2010), sheep (Ranilla et al., 1994; El Amiri et al., 2007), goats (Humblot et al., 1990; Sousa et al.,1999).

PAG determination is useful for monitoring pregnancy, because any disturbance in the fetal status, i.e. fetal death, will results in a disturbance in the placental function and the expression of placental products, such as PAG. In case of fetal mortality, the concentration of PAG will fell below the PAG level in the normal pregnant animals at the same stage of pregnancy rapidly (Breukelman et al., 2005b; Ledezma-Torres et al., 2006; Zarrouk et al., 1999).

Since the number of fetus carried by the mother has an effect on the PAG concentration, as mentioned above, in cattle (Patel et al., 1997), sheep (Ranilla et al., 1994) and goats (Chentouf et al., 2007; Sousa et al., 1999), higher PAG concentration can be used as an indicator of presence of multiple fetuses.

1.2 Milk, milk secretion and different milk treatments

Milk is a biological fluid secreted by the mammalian gland of the mammal's female, to meet the nutritional requirements of the neonate in the first stage of his life. The composition of the milk can vary between species and breeds of the same species. Individual animals, health, nutritional status, lactation stage, animal's age, intervals between milking times can have also some effect on milk composition (Huppertz and Kelly, 2009; Tambajong, 2002). Milk can be considered as a complicated mix which consist mainly of water (87.5 %), carbohydrate (mainly lactose 4.8 %), Fat (3.7%), protein (3.5 %), minerals (0.72 %) and other substances, which exist only in small quantities like vitamins, enzymes, growth factors and hormones (Grosvenor et al., 1993).

The secretory unit in the udder (the mammalian gland) is the alveoli; each alveolus is consisted of a single layer of secretory epithelial cells which enclose the alveolar lumen. This layer could be considered as a barrier to the transfer of substances from blood to the milk (Fox and McSweeny, 1998). The mammary epithelial cell controls the uptake of blood-borne molecules at its basal side and the release of products at its apical side, using mechanisms of internalization (endocytosis) and mechanisms of release (exocytosis). These mechanisms are strictly dependent on the physiological stage of the mammary gland. Mcmanaman et al. (2003) have explained that milk lipids consist mainly of triglycerides and phospholipids in the basal level of the secretory cell in the smooth endoplasmic reticulum. New formed lipid molecules form cytoplasmic lipid droplets which grow in size and moved closer to the apical plasma membrane where they are secreted into the alveolus lumen. These membrane enclosed structure are called milk fat globules. Boisgard et al. (2000) suggested that milk proteins appear over the endoplasmic reticulum, transiently associated with elements of the Golgi complex, then concentrate in post-Golgi secretory vesicles where caseins are detectable in aggregated form, the casein micelles.

According Also to Boisgard et al. (2000) the mammary epithelial cell internalizes plasma-borne proteins like hormones, growth factors, transferrin and immunoglobulins, partly via clathrin-coated vesicles, and carry many of them by transcytosis to apical region of the mammary cells, where they are released. The way in which the PAGs are being transferred from the blood to the milk is still unknown. Ali et al. (1999) assumed that the PAG as a water-soluble protein may be able to cross the surface membrane lipid bi-layer. Peaker (1974) suggested that the growth factors may use the presence of leaky tight junction between the secretory cells at the time of parturition, which facilitate their transfer to the milk; this road may be proposed for the PAG.

It has been reported that bovine milk contains many different enzymes (Got et al., 1971; Shahani et al., 1973). Those enzymes could be of indigenous origin as they are secreted normally with the milk (Fox and Kelly, 2006). Or they may be originating from some microorganisms which can contaminate the milk (exogenous enzymes). Some of the exogenous enzymes may cause some undesirable changes in the milk, e.g. hydrolytic rancidity of the milk or proteolysis (Fox and McSweeny, 1998).

The activity of the milk enzymes (endogenous and exogenous), in addition to the activity of the microorganisms present in the milk which are responsible for the milk deterioration, could be reduced or prevented by adding milk preservation materials, like hydrogen peroxide H_2O_2, mercuric chloride HgC_{12}, potassium dichromate $K_2Cr_2O_7$, sodium azide NaN_3 and bronopol $C3H6BrNO4$ (Kroeger, 1985). The addition of preservatives allows a longer storage of fresh milk but they can interfere with some analytical methods used to determine compounds and components present in milk like somatic cell count using the fossomatic® counter (Martinez et al., 2003) and Aflatoxin M1 immunoassay (Rubio et al., 2009).

Milk contains different types of hormones and growth factors such as pituitary hormones like prolactin (Malven and Mcmurtary, 1974), growth hormone or somatotropin (Torkelson, 1987). Some hypothalamus hormones, like gonadotropin-releasing hormone (GnRH), thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone (LH-RH) and somatostatin as have been reported by Baram et al. (1977), Amarant et al. (1982) and Takeyama et al. (1990), respectively. Other hormones that could be detected in milk are the gonadal hormones, especially estrogens (Wolford and Argoudelis, 1979) and progesterone (Darling et al., 1974), the determination of progesterone provided a useful tool to recognize pregnant and non-pregnant cows (Comin et al., 2005; Holtz et al., 1986). Tucker and Schwalm. (1977) showed the presence of cortisol and corticosterone in milk. Additional hormones and bioactive products like paratheroid hormone-related protein (Budayr et al., 1989), insulin (Malven, 1977) and growth factors (Campbell and Baumrucker, 1989; Malven et al., 1987) have been detected in milk.

In many analytical assays the milk fat concentrations has to be considered. In the case of liposoluble progesterone e.g., milk skimming will lead to a drastic decrease in the progesterone level measured in the milk as have been reported by Darling et al. (1974) when comparing progesterone content between milk cream and skim milk. In the case of water-soluble hormones the milk fat may play a role in scattering the light used by the photospectrometer in the case of enzyme immunoassays. The scattered light does not follow Lambert-beer law of absorbance and results in false positive or false negative results in the immunoassay (Datta and Dasgupta, 2010).

Heat treatment is the standard method for preserving milk. For pasteurization the milk container is put into a water bath until the milk reaches 63°C and holding this temperature for 30 minutes (Holder method). Another method where the milk samples would be kept at 72° C

for 15 seconds or the high temperature short time method (HTST). Milk could also be sterilized by heating the milk to a very high temperature for a very short time (138° C for 2 seconds) or as it is known as ultra–high temperature treatment (UHT), which increase the storage ability of the milk (Lewis, 2003). New methods have been suggested lately replacing thermal pasteurization like treatment with pulsed electric fields (Bendicho et al., 2002), however, this method require special complex equipment to be applied, and may be more expensive than the more standard heat treatment (Singh and Kumar, 2011).

The milk heat treatment is used to inactivate temperature-sensitive pathogenic and spoilage micro-organisms by reducing their ability to multiply and produce the milk deteriorating enzymes (Lewis and Deeth, 2009). It also affects native milk proteins and enzymes (Fox and McSweeny, 1998). Heat treatment may increase the ability to store the milk without much change throughout the storage time at refrigeration temperature (Bermudez, 2008). Pasteurisation is recognized as the main method for heat treatment; its main objective is to inactivate non-spore pathogens and reduce non-pathogenic micro-organisms which may cause milk spoilage to increase the milk stability throughout storage (Lewis and Deeth, 2009). Different heat treatments can also deactivate the indigenous and exogenous enzymes milk (Fox and Kelly, 2006; Walstra et al., 1999) and reduce the activity of bioactive proteins like the immunoglobulins or lactoferrin in different extents. By the use of ultra-high temperature (UHT) nearly all of them were inactivated (Li-Chan et al., 1995; Mancini et al., 1965; Mata et al., 1998; Paulsson et al., 1993).

The storage durability depends also on the previous processing of the milk. Lopez-Fandino et al. (1993) have reported that the proteolytic degradation during storage of UHT treated skim milk is greater than that of whole milk subjected to the same UHT treatments. According to Deeth et al. (2002) skim milk samples showed more susceptibility to proteolysis than whole milk samples when cultured with spoilage organisms after pasteurization. However, as has

been noticed by Igarashi (1990), heating the milk to pasteurization temperature did not enhance the proteolytic activity in the milk.

Treatment with ultrasound (sonication) uses sound frequencies higher than those audible by human ear. The high power (10-1000 W/cm^2) sound waves at a low frequency (20-1000 KHz) causes damage to the bacteria but may also cause changes in the physical structure of milk and the milk components, so the Ultrasound treatment can be used for milk homogenization. sonication may lead also to formation of free radicals (mainly OH- and H+), which have in way or another some effect on the bacteria and on the chemical changes in milk (Bermudez-Aguirre et al., 2009 and 2011; Cameron et al., 2009; Gera and Doores, 2011; Piyasena et al., 2003).

The effect of sonication on milk microorganisms is attributed to cavitation and shear forces, localized heating and free radical formation. Effects on the milk enzymes are also possible depending on molecular structure of the enzyme. The combination of heat treatment or high pressure or both with ultrasound treatment may induce more inactivation of the enzymes and microorganisms in the milk in a shorter period of time (Manas et al., 2000; Ordonez et al., 1984; Piyasena et al., 2003).The ultrasonic treatment could also be applied to the milk by inserting a sonicator head in the milk, or could be done by placing the milk samples containers inside an ultrasonic water bath (Mason et al., 2003).

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Chapter 2

Pregnancy-associated glycoprotein (PAG) pattern and pregnancy detection in Boer goats using an ELISA with different antisera

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Abstract

Pregnancy-associated glycoproteins (PAGs) are macromolecules produced by placental tissue and released into the maternal circulation where they allow pregnancy diagnosis and Follow-up. The present study addresses the question to what extent plasma PAG determination may serve as a means of early pregnancy detection in goats in a similar way it is practiced in cows, and whether an ovine or bovine PAG-ELISA may be utilized to this end. Blood samples were collected from eight pregnant pluriparous Boer goat does twice weekly during the first seven weeks and the last four weeks of pregnancy and weekly in-between and during four weeks following parturition. Plasma PAG concentrations (mean ± SEM) were determined using a competitive enzyme-linked immunosorbent assay. Assays were conducted with polyclonal antisera raised in rabbits against purified preparations of caprine (AS#706), ovine (AS#780) and bovine PAG (AS#726). In the assay systems purified bovine PAG served as standard and tracer and goat anti-rabbit IgG served as coating antibody. With the antibody raised against caprine PAG (AS#706) a steep increase to a climax of 69 ± 9 ng/ml on day 56 of pregnancy was followed by a gradual decline to 16 ± 3 ng/mL at parturition and 0.3 ± 0.07 ng/mL four weeks postpartum. The results achieved with the anti-ovine PAG (AS#780) showed close similarity, a maximum of 92 ± 14 ng/mL being reached at 56 days of pregnancy. With anti-bovine PAG (AS#726), the PAG level increased to a maximum of 3.1 ± 0.2 ng/mL on day 105 of pregnancy and fluctuated around 3 ng/mL until the end of pregnancy. The difference between pregnant and non-pregnant does reached a significant level 21 days after conception, one week earlier than with caprine and ovine antisera.

2.1 Introduction

The availability of a means of early pregnancy diagnosis is of practical relevance in the goat business. In cows the most common way of pregnancy detection is by rectal palpation. For morphological reasons this method is not applicable in goats. Apart from observing the return

to Estrus, the most common means of diagnosing pregnancy in goats are transrectal or transabdominal ultrasonic scanning (Martinez et al., 1998; Padilla-Rivas et al., 2005), progesterone measurement in blood or milk (Agwu and Holtz, 1986), estrogens in blood (Dhindsa et al., 1981; McArthur and Geary, 1986; Sindermann et al., 1992) or feces (Holtz, 1992; Sindermann et al., 1992; Ledezma-Torres, 2002) and, more recently, the determination of pregnancy-associated glycoprotein (PAG) in blood (Sousa et al., 1999; Gonzalez et al., 1999; Batalha et al., 2001) or milk (Gonzalez et al., 2001). PAG may be measured by radioimmunoassay (RIA) (Sasser et al., 1986; Zoli et al., 1992) or enzyme-linked immunosorbent assay (ELISA) using monoclonal (Green et al., 2005) or polyclonal antibodies (Friedrich and Holtz, 2004, 2010).

The aim of the present study was to (a) establish a PAG pregnancy profile for Boer goats, (b) determine from what stage of gestation onward plasma PAG may serve as a reliable diagnostic tool and (c) establish whether PAG in goat serum may be detected by an assay based on antibodies raised against ovine or bovine PAG.

2.2 Material and methods

The investigation was conducted on Boer goats of the departmental flock of Goettingen University. The animals were group housed in open barns with straw bedding and outdoor concrete runs. Does were fed a daily ration of 600 g concentrate, consisting of equal parts of a pelleted diet for breeding ewes (16% crude protein, 12.2 MJ metabolizable energy/kg, supplemented with Se, I and Zn), oats and dried sugar beet pulp and had free access to straw, salt lick and water. From eight pregnant does blood samples of 4 ml were drawn by jugular venipuncture twice weekly during the first seven and the last four weeks of pregnancy and weekly in-between and during four weeks following parturition. By way of comparison, blood samples were drawn from nine non-inseminated does twice weekly for seven weeks after estrus. Collecting tubes contained three drops of sodium citrate to prevent clotting. After

centrifugation at 2000 × g for 10 min at 4 °C, plasma was stored at −20° C until being assayed. Three ELISA systems were used to measure PAG concentration in Boer goats. Plasma concentrations of PAG were first determined by homologous competitive enzyme-linked immunosorbent assay (ELISA) in the way described in Friedrich and Holtz (2010). Briefly, PAG antiserum AS#706 raised against a purified caprine PAG preparation (caPAG55+62 kDa; Garbayo et al., 1998) served as specific antibody, whereas purified bovine PAG (boPAG67 kDa, Zoli et al., 1991) was used as standard and tracer. Two additional heterologous polyclonal antibodies (named AS#780 and AS#726), raised against ovine PAG (ovPAG57+59 kDa; El Amiri et al., 2003) (AS#780) and bovine PAG (boPAG67 kDa) (AS#726), respectively, were used. The respective antisera were diluted in assay buffer (0.1% casein, 0.005 M NaOH, 0.12 M NaCl, 0.02 M Na2HPO4, 0.01 M EDTA, 0.002% phenol red, 0.005% chlorhexidine digluconate (20%), pH 7.3) at a ratio of 1:200,000 (AS#726), 1:320,000 (AS#706) and 1:80,000 (AS#780), respectively. Volumes of 100 µL/well were added to goat anti-rabbit coated microtiter plates (Nunc Maxisorp®, Thermo fisher, Germany). The plates were incubated overnight at 4°C. Standard curves were prepared from purified bovine PAG diluted in PAG-free serum at concentrations of 0.0, 0.39, 0.78, 1.56, 3.125, 6.25 and 12.5 ng/mL, respectively.

Of the tracer (biotinylated boPAG67 kDa, diluted 1:1000 in assay buffer), 50 µL was added to each well, followed by 90 min of incubation at room temperature. After two washings (washer: Columbus Plus, Tecan, Germany)100 µL/well streptavidin-peroxidase (50 µg/mL) and, after four more washings, 150 µL/well 3,3´,5,5´-tetramethylbenzidine (12.5 mg/mL DMSO,Sigma) were added, followed by 30 min incubation at room temperaturein the dark. Optical density was measured by Tecan Sunrise® photometer with software MAGELLAN 4.0 (Tecan) at wave length 450 nm. Concentrations were calculated using a logit-log transformation according toRodbard (1974).

Means and SEM concentrations were calculated using Proc means inSAS 9.1 software (SAS institute Inc., Cray, NC). Using software JMP IN(6.0.0), PAG concentration of pregnant and non-pregnant animals was compared using Dunnet's t-test, whereas the difference in PAG concentration of various antisera at different stages of pregnancy and post partum period was tested for significance by Student's t-test.

2.3 Results

The PAG profile of eight pregnant goats (two bearing singletons, five bearing twins and one bearing a triplet) established with an ELISA based on an antiserum raised against caprine PAG was characterized by a rapid increase to a climax of 69 ± 9 ng/mL arrived at 56 days after conception, followed by a gradual decline to 16 ± 3 ng/mL at parturition and 0.3 ± 0.07 ng/mL four weeks postpartum (Figure. 1).

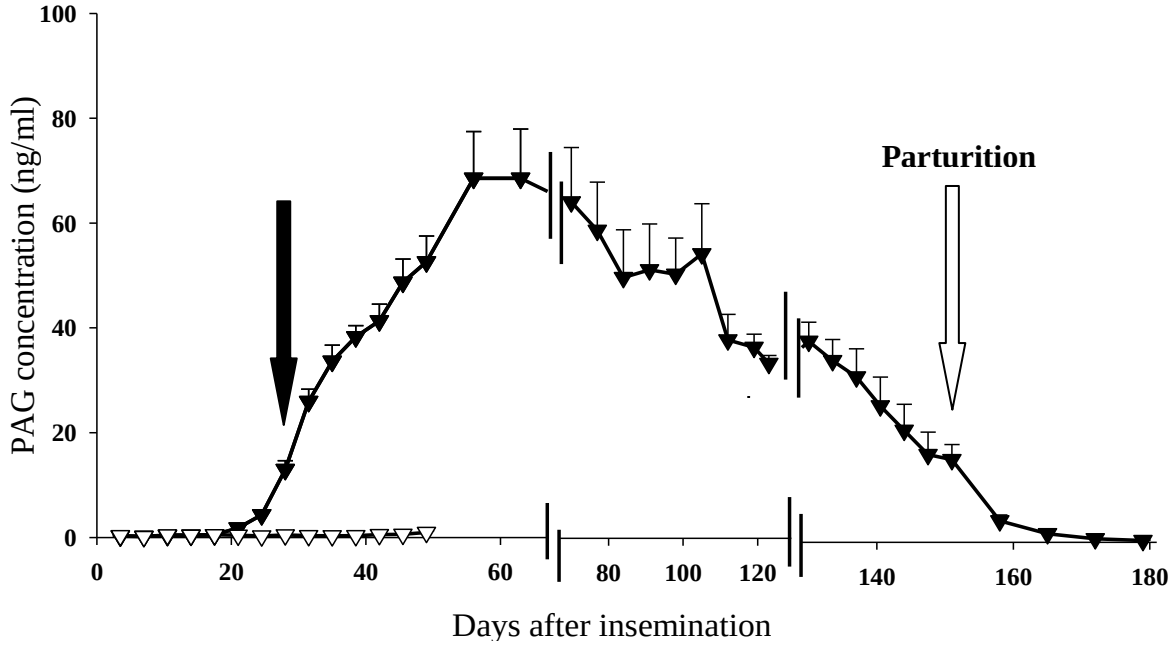

Figure 1. PAG profile (mean ± SEM) of 8 pregnant (closed triangles) and 9 non-pregnant (open triangles) Boer goat does assessed by an ELISA based on caprine antiserum raised against caprine PAG (AS#706). Data are arranged around the times of mating and parturition. The black arrow signifies the point at which pregnant and non-pregnant does differed significantly (P < 0.05)

The plasma PAG concentration of the singleton bearing does was between 25% and 40% (at the climax of the curve) below that of does bearing multiple fetuses. The PAG profile established when using antiserum raised against ovine PAG closely resembled that obtained with antiserum raised against caprine PAG, though at a slightly higher level (Figure. 2).

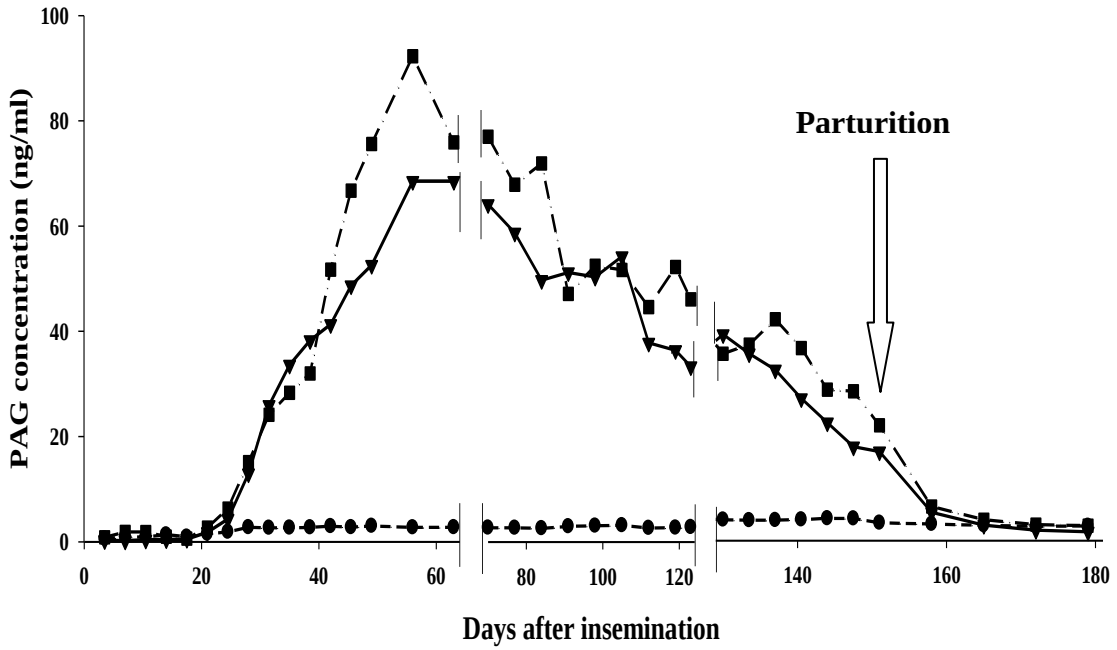

Figure 2. Mean PAG profiles of 8 pregnant Boer goats does assessed by ELISAs based on antisera raised against caprine (AS#706, triangles), and ovine (AS#780, squares) and bovine PAG (AS#726, circles). Data are arranged around the times of mating and parturition.

A peak value of 92 ± 14 ng/mL was reached on day 56. The curves only differed significantly on days 49, 56 and 84 of pregnancy ($P < 0.05$). When using an assay system based on antiserum raised against bovine PAG, levels resembled those of the other tests until the second week of pregnancy. The subsequent increase, however, was rather modest; a maximum of 3.1 ± 0.2 ng/mL was reached on day 105 (Fig. 2). When changing the scale of the ordinate (Fig. 3) it became evident that, with antiserum raised against bovine PAG, the pattern differed from that observed when using caprine or ovine antisera. After an initial

increase between days 14 and 28 of pregnancy, the PAG concentration fluctuated around a value of 3 ng/ml until parturition without a marked increase and declined gradually thereafter.

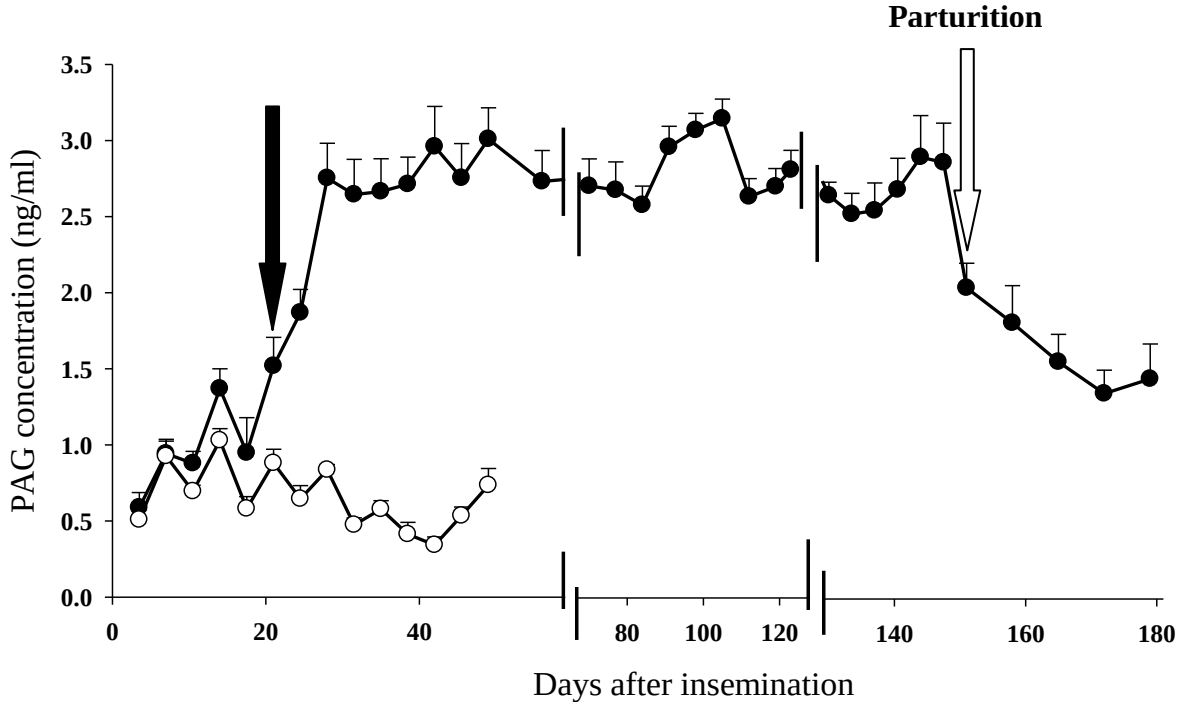

Figure 3. PAG profile (mean ± SEM) of 8 pregnant (closed circles) and 9 nonpregnant (open circles) Boer goat does assessed by an ELISA based on antiserum raised against bovine PAG (AS#726). Data are arranged around the times of mating and parturition. The black arrow signifies the point at which pregnant and non-pregnant does differed significantly (P < 0.05).

Table 1 describes parameters characterizing the different PAG patterns. When using antiserum raised against caprine and ovine PAG the difference between pregnant and non-pregnant does reached significance levels on day 28. When using antiserum raised against bovine PAG, much lower levels were recorded, but the difference between pregnant and non-pregnant animals was significant as early as day 21 of pregnancy ($P < 0.05$).

Table 1 . Characterization of plasma PAG determination in goats using antisera raised against caprine (AS#706), ovine (AS#780) and bovine (AS#726) PAG.

Variable	CaprineAS	OvineAS	BovineAS
Detection level (ng/mL)	0.15 (SEM 0.01)	0.30 (SEM 0.04)	0.33 (SEM 0.04
Basal level (ng/mL)	0.21 (SEM 0.02)	0.61 (SEM 0.13)	0.58 (SEM 0.09)
Maximum concentration (ng/mL)	69.00 (SEM 9.00)	92.00 (SEM 13.70)	3.10 (SEM 0.20)
Return to basal level (days p.p.)	30	>30	>30
Earliest pregnancy diagnosis (day)[a]	28	28	21

[a] the point in time when the difference between pregnant and non-pregnant doesValues differ is significantly from those of non-pregnant does ($P < 0.05$)

2.4 Discussion

The plasma PAG profile recorded when using an assay based on anti-caprine PAG resembles that reported for goats by Batalha et al. (2001) and Gonzalez et al. (2000). Slight differences, e.g. a second maximum between the 17th and 19th week of gestation, reported for Brazilian Caninde goats by Sousa et al. (1999), are possibly breed related or based on the use of different antisera that bind to different PAG isoforms. Gonzalez et al. (2000) have reported significantly different PAG patterns when using RIA systems with different anti-caprine PAG antisera. The differences in PAG concentration between our study and studies by others may result from different affinities of the polyclonal antisera toward different PAG isoforms. According to Xie et al. (1993, 1995) and Garbayo et al. (1998, 2000) different genes express different isoforms of PAG at different stages of gestation. Therefore, in all likelihood different main PAG isoforms are expressed during different stages of pregnancy. In order to precisely

identify expression patterns in the course of pregnancy it would be necessary to extract PAG from placental tissues collected at different stages of gestation. The difference in PAG level between does bearing singletons versus does bearing multiple kids was evident but due to the small numbers (two versus six does) statistical verification was not attempted. The results obtained with antiserum AS#780, raised against ovine PAG, are in agreement with those reported by other investigators using the same antiserum to measure PAG concentrations in goats (Sousa et al., 1999; Zarrouk et al., 1999; Batalha et al., 2001). Due to the similarity in PAG pattern obtained with antisera raised against caprine and ovine PAG, it is admissible to measure PAG concentrations in goats in an ovine assay system. Conversely, as shown by Ledezma-Torres et al. (2006), ovine PAG may be detected in a caprine assay system. With bovine antiserum an altogether different pattern was recorded and the pregnancy-related increase was far less conspicuous. Nonetheless, the difference between pregnant and non-pregnant does reached significance levels on as soon as 21 days after conception.

2.5 Conclusion

With the aid of an ELISA based on antibodies raised against caprine or ovine PAG it is possible to reliably diagnose pregnancy in Boer goats from day 28 of pregnancy onward. In an assay based on antibodies raised against bovine PAG, despite a moderate increase and deviating pattern, pregnancy may be detected by day 21. This implies that it is permissible to include blood samples from goats with routine pregnancy testing conducted in cows with no extra effort and expense.

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Chapter 3

Pregnancy-Associated-Glycoprotein (PAG) profiles in dairy, dual purpose and beef cattle

Abstract

Pregnancy-associated glycoproteins (PAG) are produced by mono- and binucleate trophoblast cells in the ruminant placenta. PAG appears in maternal blood and, from about four weeks after fertilization onward, may serve as a reliable means of diagnosing pregnancy. A range of factors are said to affect plasma PAG concentrations, such as number and sex of fetus, mass of calf and placenta, level of milk production and genetic constitution. In the present study PAG pregnancy profiles of a dual-purpose (Simmental) and two beef breeds (Uckermark and Aubrac) are compared with the profile of the specialized dairy breed Holstein-Friesian. Holstein-Friesian cows were sampled weekly; the levels of the other breeds were presented at three-week intervals. The overall significant breed difference ($P=0.013$) was founded on deviations during the initial three weeks of pregnancy and from 23 weeks onward. During the period critical for the detection of pregnancy, between four and 22 weeks, agreement between PAG levels of various breeds was close ($P>0.05$). No significant effect of body mass of cow or calf (relative to mass of dam) was detected. These findings imply that the PAG pregnancy test may be executed irrespective of breed or type of cow, affirming the suitability of the test as a valuable asset for the cattle industry.

3.1 Introduction

Pregnancy-associated glycoproteins (PAGs) are aspartate proteases (Xie et al., 1991) synthesized by mono- and binucleate cells of the trophectoderm in ruminants (Wooding et al., 2005). PAGs appear in maternal blood shortly after implantation and are detectable throughout gestation. Their presence indicates the existence of a functional placenta and a viable embryo (Ranilla et al., 1994; Sousa et al., 1999; Zoli et al., 1992). Radioimmunoassay (Zoli et al., 1992) and enzyme-linked immunosorbent assays (Friedrich and Holtz, 2004 and 2010; Green et al., 2005) can be used to reliably diagnose pregnancy in cows from Day 28 of

pregnancy onward. The intention of the present investigation was to present a representative serum PAG profile for Holstein Friesian cows by sampling of ten cows weekly throughout a complete pregnancy period and to compare the pattern of the Holstein Friesian breed with that of a dual purpose and two beef breeds.

3.2 Material and methods

Weekly blood samples were drawn via caudal venipuncture from ten lactating pluriparous Holstein-Friesian cows throughout a complete pregnancy period. These cows had an average performance of 8500 kg and were housed in a free stall barn under intensive husbandry conditions. In addition blood samples were drawn from 22 Simmental, 21 Aubrac and 20 Uckermark cows. Simmental and Aubrac cows were kept at the experimental farm Relliehausen of Goettingen University under extensive pasture conditions during summer and in a free stall barn in winter; Uckermark cows were stationed at an experimental farm of "Landesamt fuer Verbraucherschutz, Landwirtschaft und Flurordnung" (LVLF) Grosskreutz and were kept outdoors year round. Under the extensive husbandry conditions samples were drawn at six week intervals and grouped three-weekly. In Uckermark cows sampling was terminated after the 210th day of pregnancy for technical reasons. Whereas Holstein-Friesian cows were artificially inseminated, Simmental, Aubrac and Uckermark cows were naturally mated by bulls running in the herd. The date of service was calculated from the calving date, assuming a pregnancy period of 285 days, typical for those breeds. Blood samples were kept on crushed ice until reaching the laboratory. After four hours at room temperature they were centrifuged for 10 min at 2000 x g. Serum was stored at -20°C and assayed for PAG content by polyclonal ELISA described in detail by Friedrich and Holtz (2010). In Simmental and Aubrac cows, life weights of cows and calves were recorded at the time of parturition. Means and standard deviations of PAG concentrations were calculated using MS Excel®. The fixed effects of breed and pregnancy period were tested using *Proc MIXED* with repeated

measurements using SAS 9.3 package (SAS Institute, Cary, NC, USA) after logarithmic transformation of the data and considering individual animal a random factor. To compare PAG patterns of different breeds the pregnancy curve was divided up into the segments 0 to 3 weeks, 4 to 12 weeks, 13 to 21 weeks and 22 to 35 weeks.

3.3 Results

Serum PAG concentrations obtained from10 Holstein-Friesian cows that were sampled at weekly intervals throughout a pregnancy period are presented in Fig. 1. From basal levels of 0.5±0.1 ng/mL prevailing during the first two weeks of pregnancy the concentration rose to 1.1±0.2 ng/mL after three and 4.4±0.5 ng/mL after five weeks (Fig. 2). After a more gradual incline to a level of 24.7±3.1 ng/mL by 21 weeks, an accelerated increment to 148.1±22.6 ng/mL at 36 weeks and a final surge climaxing at 547±104 ng/mL at parturition were recorded.

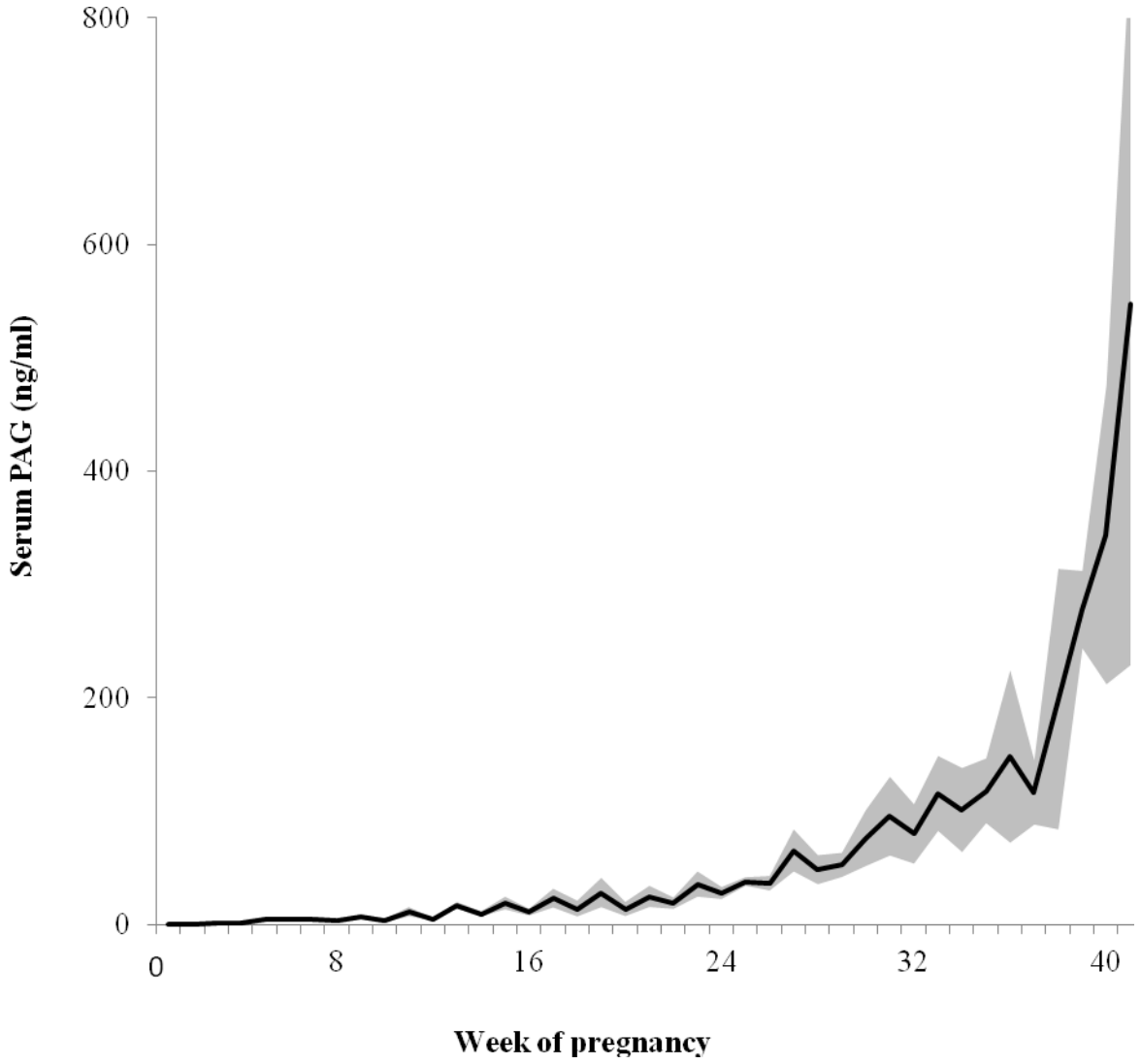

Figure 1. PAG pregnancy profile (means ± SEM) of 10 Holstein-Friesian cows sampled at weekly intervals from insemination to parturition.

Data from 19 Simmental, 19 Aubrac and 17 Uckermark cows that had calved within the expected time were included in the statistical analysis. An overall statistically significant breed effect on PAG level was found (*P=0.013*). Whereas the PAG profile of Aubrac cows closely follows that of Holstein-Friesian cows (*P>0.05*), significant deviations occurred in Simmental and Uckermark cows during the first three weeks and between the 22nd and 35th week of pregnancy (Fig. 2). Three weeks after mating the PAG concentration in Simmental

cows was 2.7 ng/mL and in Uckermark cows 1.8 ng/mL higher than that of Holstein-Friesians (both *P<0.01*). During the period from four weeks to 21 weeks of pregnancy no breed effect was manifest (*P>0.05*), however, between the 22nd and 35th week average concentrations in Simmental cows were 34.3 ng/mL and in Uckermark cows 48.0 ng/mL lower than in Holstein-Friesian cows (both P<0.01). The peripartal PAG surge in Simmental (1886±209 ng/mL) and Aubrac cows (693±82 ng/mL) was of a higher magnitude than in Holstein-Friesian cows (547±100). Due to substantial individual variability differences in peak values were not statistically significant (*P>0.05*). No data for the final surge were available for Uckermark cows because sampling had been terminated after 210 days of pregnancy.

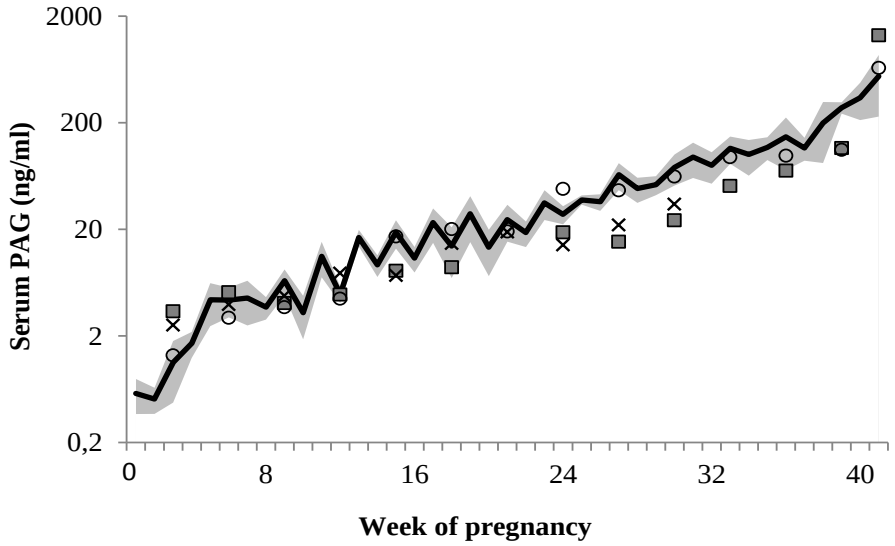

Figure 2. Semi-logarithmic presentation of PAG pregnancy profiles from Holstein-Friesian (means ± SEM), Simmental (squares), Aubrac (circles) and Uckermark cows (X; records extend only to 30 weeks).

Body weight of Simmental cows was 697±15 kg and of Aubrac cows, 612±15 kg; corresponding birth weights of calves were 52±1 kg and 42 ±1 kg, respectively.

3.4 Discussion

The PAG pattern of the Holstein-Friesian cows that were sampled weekly throughout a complete pregnancy period, as depicted in Fig. 1, resembles descriptions by Sasser *et al*. (1986), Mialon et al. (1993), Green et al. (2005) and others who did, however, not cover an entire pregnancy period on a substantial number of animals. For the characteristic pattern as yet no plausible explanation is available as nobody is certain what physiological functions PAGs may have or if they just happen to be metabolic spin-offs or excretory products.

We wanted to know to what extent the PAG-patterns of dual purpose and beef breeds agree with those of Holstein-Friesian cows and whether the same assay and threshold values may be utilized when relying on PAG concentration as an indicator of pregnancy. Holstein-Friesians, being the most widely distributed cattle breed worldwide, are a highly specialized dairy breed. German Holsteins produce, on average, 9097 kg milk/year (German Holstein Association, 2012). Simmentals, being prevalent in most of Southern Germany, Austria and Switzerland, have an average yearly milk yield of about 7100 kg (ASR, 2011). The dual purpose type Simmental cow does not produce quite as much milk but features excellent carcass traits. Their body mass slightly exceeds that of German Holsteins. Uckermark and Aubrac are specialized beef breeds. The Uckermark breed originates from Eastern Germany, where it was derived by crossing Charolaise with German Simmental. Cows weigh 750 to 850 kg, slightly more than Simmental cows. They produce just enough milk to raise a calf. The Aubrac breed originates from the central highland of France where it is kept for beef production under extensive pasture conditions. Aubrac cows are about similar in weight than Holstein-Friesian

cows and produce, on average, 2200 kg milk/year. Apart from breed differences, Holstein Friesian cows were milked twice daily until being dried off, whereas cows of the other breeds under investigation were suckling calves. In spite of an overall significant breed difference, by and large the PAG patterns displayed by the non-dairy breeds resembled that of Holstein Friesian cows. Whereas significant deviations were evident during the first three weeks, between the 22nd and 35th week and during the periparturient surge, between the third and 22nd week breed differences were not significant. This happens to be the period of interest when diagnosing pregnancy. The deviations in PAG level during the first three weeks and between 23 and 36 weeks as well as the peripartal surge are, though statistically significant, irrelevant from a diagnostic point of view. The lower serum PAG concentration observed in Holstein-Friesian cows during the first three weeks may be associated with a higher metabolic rate in dairy cows producing more than 25 liters of milk per day instead of merely the amount required to suckle a calf. This interpretation was also proposed by Mialon et al. (1993) and Lopez-Gatius et al. (2007) who reported a negative correlation between milk yield and plasma PAG concentration. On the other hand, it does not appear unlikely that residual PAG from the previous pregnancy might be partially responsible for the higher level. After all, in Simmental and Aubrac cows periparturient PAG levels reached substantially higher amplitudes than in Holstein Friesian cows. Furthermore, they were exposed to breeding bulls as soon as 60 days after calving. As a consequence, 37% of the Simmental cows, and 41% of the Uckermark cows fell pregnant less than 75 days after calving. Insemination in Holstein Friesian cows, on the other hand, was carried out more than 80 days post-partum. The half-life of PAG, according to Kiracofe et al. (1993), amounts to 8.5 days. The higher amplitudes of the terminal PAG surge recorded for Simmental and Aubrac cows, as compared to Holstein-Friesians, corresponds with reports by Zoli et al. (1992), Mialon et al. (1993) and Lobago et al. (2009) who found more prominent periparturient surges in beef cattle (Hereford, Charolais

and Borana-Holstein crosses) than in typical dairy cows (Holstein-Friesians and Race Normande).

Apart from breed and stage of pregnancy, in the literature other effects on PAG levels are mentioned, such as the number of fetuses (Dobson et al., 1993; Patel et al., 1995 and 1997), fetal sex (Zoli et al., 1992), placental mass (Echternkamp et al., 1993), birth mass of calf (Vasques et al., 1995) and amount of milk produced (Lopez-Gatius et al., 2007). Furthermore, the genetic background of the fetus was found to be responsible when cows were bred with sires of a different breed (Serrano *et al*., 2009) or served as recipients of unrelated embryos (Guilbault et al., 1991; Zoli et al., 1992). In the present study no significant relationship of PAG level with body mass or birth mass of calf (relative to mass of dam) was detected. Unpublished data from our laboratory on Holstein-Friesian cows revealed that, while birth mass of calf had no effect, sex of calf did: average PAG levels in cows bearing male calves were significantly higher than in cows with female offspring (1378 vs. 720 ng/mL, $P<0.05$).

3.5 Conclusion

In conclusion, the PAG pregnancy patterns of Simmental, Aubrac and Uckermark cows suckling their calves closely resemble the profile established for Holstein Friesian dairy cows that are being milked. Existing deviations in some of the breeds under investigation do not comprise the pregnancy period relevant for early pregnancy detection. The practical implication is that the same assay system and threshold values may be utilized for various breeds, emphasizing the potential relevance of the PAG pregnancy test for the cattle industry.

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Chapter 4

Pregnancy-associated glycoprotein (PAG) in milk of dairy cows

Abstract

Bovine milk is a composite fluid. Its concentrations of fat and protein are affected by several factors. During pregnancy traces of a placental glycoprotein, the pregnancy-associated glycoprotein, can be detected in the milk of pregnant cows using a polyclonal PAG ELISA developed by Friedrich and Holtz (2010). The current study was conducted to investigate the effect of milk preservatives, milk fat and different milk treatments on PAG concentrations in milk measured by that PAG ELISA. The effect of milk preservatives, milk type and storage duration was investigated using fresh milk (non-homogenized, non-pasteurized), organic milk (non-homogenized, pasteurized) and ultra-high temperature treated (UHT) milk (homogenized, sterilized) with 3.5 % fat. The milk was skimmed by centrifugation and treated either with bronopol, sodium azide, potassium dichromate or left without treatment as a control. Known PAG concentrations were added 2 days, 1 day and shortly before the assay. The effect of milk fat content was studied using UHT milk with two different fat contents (1.5 and 0.3 %) spiked with known concentrations of PAG. The effect of pasteurization and sonication were tested using pooled milk from dairy cows after about 2 weeks of calving. Following milk skimming, samples were pasteurized at 63°C for 30 minutes, sonicated or left without treatment as a control. Samples were then stored at three different temperatures (room temperature, 4°C and -20°C) for different durations (1, 3, 5 and 7 days). In the first experiment, fresh and UHT milk showed the best correlation coefficients between measured and expected PAG concentrations. All milk samples can be stored up to three days before the PAG analysis without drastic change in the measured PAG content. When milk preservatives were added, bronopol gave the best correlation between expected and measured PAG values. In the second experiment, differences in PAG recovery rates between different fat contents of UHT milk were statistically not significant ($P>0.05$). Recovery rates at lower PAG concentrations were significantly higher ($P<0.05$) than those with higher PAG content, and fresh prepared samples were significantly higher ($P<0.05$) than those stored for 1 or 2 days.

The effect of ultrasonic treatment was less destructive to milk PAG content compared to pasteurization, although the results in the pasteurization group were more uniform throughout storage time. The cooled milk samples did not show drastic change in the recovery rates in the first 3 days of storage.

To determine PAG concentration in milk samples using the PAG ELISA, skimmed fresh or skimmed UHT milk was preferred for the preparation of assay standards and control. When considering milk preservatives, bronopol was found to be the most desirable. The milk samples could be pasteurized and/or kept cooled for 3 days without noticeable changes in PAG content.

4.1 Introduction

Pregnancy-associated glycoproteins (PAGs) belong to the family of aspartate proteases (Xie et al., 1991). They are synthesized by the mono- and binucleate cells of the trophectoderm in the synepitheliochorial placenta in ruminants (Wooding et al., 2005). PAGs appear in maternal blood shortly after implantation and are present throughout gestation. Their presence indicates a functional placenta and a viable embryo (Ranilla et al., 1994; Sousa et al., 1999; Zoli et al., 1992). Radioimmunoassay (Zoli et al., 1992) and enzyme-linked immunosorbent assays (Friedrich and Holtz, 2010; Green et al., 2005) can be used reliably to diagnose pregnancy in various ruminants.

Detection of PAGs in milk of cows has been reported by Metelo et al. (2004) and Tainturier et al. (1996). According to Gonzalez et al. (2001), PAG concentrations in milk of dairy goats are high enough to be utilized as a means of diagnosing pregnancy from day 32 of pregnancy onward. Friedrich and Holtz (2010) found that the concentration of PAG in cows` milk amounts to 3 to 8 % of that measured in blood and that it may serve as an indicator of pregnancy from day 150 of pregnancy onwards. Individual variations were substantial, conceivably associated with varying milk composition or milk treatment prior to assaying. A reliable measurement of PAG in full-cream milk is not possible because of the variability of its contents and its strong matrix effects on ELISA systems.

The synthesis of milk, especially colostrum, involves active and selective transcytotic processes (Ollivier-Bousquet, 2002). Some blood serum components, such as IgG in colostrum (Watson, 1980) or progesterone are accumulating in milk (Heap et al., 1973). Others, such as PAG, are transferred to the milk to a lesser degree (Friedrich and Holtz, 2010).

The aim of this study was to explore the effects of milk preservatives, milk fat content and milk processing on the outcome of PAG measurements using a polyclonal PAG-ELISA. This is necessary for further studies, in which milk samples are collected in the field and cannot be analyzed shortly after collection and have to be shipped or stored.

4.2 Material and methods

PAG concentrations were determined in milk samples using a homologous competitive enzyme-linked immunosorbent assay (ELISA) which was described earlier (Friedrich and Holtz, 2010). Briefly, milk samples were diluted 1:1 in assay buffer and purified bovine pregnancy-associated glycoprotein-1 was used as standard and tracer. Polyclonal antiserum raised against bovine PAG (AS726) was used as specific antiserum. Concentrations were calculated using a logit-log transformation.

4.2.1 Experiment 1: Effect of milk preservatives, milk type and storage duration

Three different types of milk were used in this experiment: a) Non-pasteurized and non-homogenized, b) pasteurized and non-homogenized and c) pasteurized and homogenized. These were: a) fresh dairy bulk milk, obtained from a dairy farm four hours after milking, b) commercially available “organic” milk and c) commercially available UHT milk. All types of milk had a fat content of approximately 3.5 %. The different types of milk were centrifuged in 40 mL tubes at 620 x g for 40 minutes to remove milk fat. Skimmed milk from each type of milk was divided into four preservative treatment groups with 30 mL aliquots. To each

aliquot, either 1.5 µL/mL bronopol (2-bromo-2-nitropropane-1, 3-diol), 2 µL/mL sodium azide or 1 mg/mL potassium dichromate was added. The fourth aliquot was left untreated and served as a control.

From each aliquot 1000 µl of milk were used to prepare dilution curves with known PAG concentrations. In doing so, serum containing at least 1000 ng/mL PAG from 10 cows was pooled and added such that PAG concentrations of 0.39, 0.78, 1.56, 3.125, 6.25 and 12.5 ng/mL were obtained.

Besides the effect of the milk type and the preservative treatment, the effect of storage duration was also determined. To do this, milk samples were prepared for each type of milk and each treatment either two days, one day and shortly before being assayed. All samples were stored at room temperature (23°C). The experiment was conducted with three replications.

4.2.2 Experiment 2: Effect of milk fat, pasteurization, sonication and storage temperature

In the first trial of this experiment, to prepare milk samples with known concentrations of PAG, available blood serum from cows at an advanced stage of pregnancy was added to ultra-high temperature treated (UHT) milk with 0.3 or 1.5 % fat content. Blood was obtained via jugular venipuncture, stored at 4°C overnight and centrifuged for 10 minutes at 2000 x g. PAG concentration in serum was determined by ELISA as described by Friedrich and Holtz (2010). Sera (containing at least 1000 ng/mL PAG) from 10 cows were pooled and added to milk such that PAG concentrations of 0.25, 0.5, 1, 2, 4 and 8 ng/mL were obtained. This took place 2 days, 1 day and shortly before the milk samples were assayed and storage was at 4°C. This trial was replicated three times.

In the second trial, in order to study the effect of pasteurization, sonication and storage temperature, milk samples (12 mL) were obtained two hours after milking from 4 pluriparous cows two weeks after calving. Within 6 hours after collection, samples were pooled and centrifuged for 30 minutes at 620 x g. twelve aliquots of 2 mL skimmed milk were transferred to 5 mL test tubes and treated as follows.

Four vials were pasteurized for 30 minutes in a water bath at 63°C. Another four vials were inserted into holes in a styrofoam block floating on a sonication bath (Sonorex RK-31, Bandeline, Germany) and sonicated for five minutes at 120 W and frequency of 35 KHz. The remaining four vials were left untreated to serve as control.

After the respective treatments, each of the 2 mL milk aliquots was divided into 500 µL-aliquots, which were kept at 23°C (room temperature), +4°C or -20°C for one, three, five or seven days, respectively. Eventually, all samples were analyzed for PAG as mentioned above. This trial was conducted with three replications.

4.2.3 Statistical analysis

In all experiments, results were expressed as percentages of recovery rate averages. Mean PAG concentrations and standard deviations were calculated using MS Excel®. Concentrations of 0.39 and 12.5 ng/mL were excluded in the analysis of the 1st experiment due to the very high recovery rates and very high SD. whereas, the recovery rates in the case of 0.25 and 0.5 ng/mL in first trial of the second experiment was omitted from the calculation due to the same reason mentioned previously.

In the 1st experiment, the correlation coefficients between expected and measured PAG concentrations were calculated using a linear regression model of SPSS 16.0 software for Windows (SPSS Inc.,Chicago, IL, USA).

In the 2nd experiment first trial, mean PAG recovery rates were calculated for the PAG concentrations within the storage duration. The calculated recovery rates were based on the expected amount of PAG for each dilution. The effect of milk fat content and storage day on the PAG recovery rates was evaluated using *proc mixed* procedure of SAS 9.3 package (SAS Institute, Cary, USA) with milk fat content, storage day and PAG concentration included as fixed effects in the model, milk sample was considered as the random effect in the model.

In the 2nd experiment second trial , the effect of milk treatment (pasteurization, sonication and control), storage day and storage temperature on the PAG recovery rates was evaluated using *proc mixed* procedure of SAS 9.3 package (SAS Institute, Cary, USA) with milk treatment, storage day and storage temperature included as fixed effects and milk sample as random effect in the model.

4.3 Results

4.3.1 Experiment 1: Effect of milk preservatives, milk type and storage duration

The correlation coefficients between the expected and measured PAG concentrations according to milk type, storage duration and milk preservatives are presented in tables 1 and 2. Fresh and UHT milk showed higher correlation coefficients (0.9 and 0.791, respectively) in comparison with organic milk (0.765). These results imply that fresh and UHT milk represent a better choice to prepare the control and standard samples. Moreover, in samples that did not receive any preservatives, measured PAG concentrations in UHT milk samples showed high correlation with their counterparts in fresh milk.

Table 1: Correlation coefficient (R) between the expected and measured PAG concentrations in different types of milk, different preservatives and different storage durations.

milk type	R
organic	0.765
fresh	0.791
UHT	0.9

Preservative	R
Bronopol	0.861
Sodium azide	0.838
potassium Dichromate	0.827
without preservative	0.739

storage day	R
1	0.757
2	0.893
3	0.882

The correlation between expected and measured PAG concentrations throughout the storage time was always above 0.75. This shows the possibility of storing milk samples for up to 3 days without drastic change in PAG content.

When the correlation between expected and measured PAG concentration in the milk samples treated with preservatives were compared, the highest correlation coefficient was noticed in

bronopol treated samples. Correlation coefficients in the samples treated with sodium azide and potassium dichromate were lower. In addition, the correlation coefficient between bronopol-treated fresh milk samples and bronopol-treated UHT milk samples was higher than that between bronopol-treated fresh milk samples and bronopol-treated organic milk samples. This suggests that bronopol was the most desirable milk preservative.

Table 2: Correlation coefficient (R) between PAG concentrations measured in fresh milk and their counterparts in organic and UHT milks when treated with different preservatives.

Bronopol	**R**
organic with fresh	0.906
UHT with fresh	0.883
sodium Azide	R
organic with fresh	0.884
UHT with fresh	0.859
Potassium Dichromate	R
organic with fresh	0.865
UHT with fresh	0.268
without preservative	R
organic with fresh	0.208
UHT with fresh	0.889

4.3.2 Experiment 2: Effect of milk fat, pasteurization, sonication and storage temperature

In the first trial of the second experiment, milk samples kept at +4°C for one or two days beforehand showed a tendency for recovery rates to be lower (124 % and 113 %, respectively, P=0.05) than those prepared fresh before the assay (140 %). Statistical analysis showed a significant effect of storage duration and the added PAG levels (P<0.05) while no significant effect of fat content could be found (P=0.74). Additionally, there also was a non-significant tendency for recovery rates to be lower as the added PAG levels were increased, except for the highest concentration (8 ng/mL) where the trend was reversed.

Table 3: PAG recovery rates and SD as percent of PAG concentrations in the control samples of fresh cow´s milk after different treatment, storage temperature and storage durations.

Treatment	Storage Temp. (°C)	Storage duration (days) 1		3		5		7	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Pasteurization	**+23**	89	17	88	26	116	21	87	19
	+4	86	9	85	20	117	44	93	28
	-20	76	14	86	19	109	32	96	17
Sonication	**+23**	92	26	137	97	130	37	112	37
	+4	93	10	96	38	136	36	130	67
	-20	97	8	94	21	116	17	107	40
Control	**+23**	100		142	99	145	43	116	30
	+4	94	20	106	26	155	48	141	53
	-20	107	16	104	22	124	22	126	20
Overall		**93**	**15**	**104**	**41**	**127**	**33**	**112**	**34**

The effects of pasteurization, sonication and storage temperature on recovery rates and standard deviations of the PAG concentrations are presented in tables 3 and 4. Mean PAG levels in control samples kept at room temperature on the first day of storage was used as a reference value for recovery rate calculations.

Table 4: Mean recovery rates and SD as percent of fresh cow`s milk PAG concentrations after different treatments and storage temperatures.

Treatment	Storage temperature (°C)							
	+23		+4		-20		Total	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Pasteurization	95	21	95	25	92	21	94	22
Sonication	118	49	113	38	104	21	112	36
Control	126	57	124	37	115	20	122	36
Overall	**113**	**42**	**111**	**33**	**103**	**21**	**109**	**32**

As shown in table 3, the total recovery rates for each day of storage increased from 93 % at the 1st day to 127 % on 5th day. Mean standard deviation rose from 15 % on day 1 to 41 % on 3rd day and stayed high until 7th day. Significant differences were noticed between PAG levels at 1st and 5th day (P=0.007), and 1st and 7th day of the storage (P=0.009). The effects of milk treatments and storage duration on the PAG content in milk were significant (P< 0.05). While the mean recovery rates for the control and the sonication groups were 122 % and 112 %, respectively, the mean recovery rate after pasteurization was significantly lower (94 %, P<0.05). The temperature during storage had no significant effect on the recovery rates, although the highest variations in the recovery rates were observed in samples stored at room temperature. The interaction of storage day, storage temperature and treatment had no significant effect (p> 0.05) on PAG content in milk.

Generally, the mean recovery rates in milk samples that had been kept cooled stayed at the same level during storage. While the variation within each treatment increased, especially in samples stored at room temperature (control).

4.4 Discussion

4.4.1 Experiment 1: The Effect of milk preservatives, milk type and storage duration

Considering the effect of milk preservatives on milk ELISA results, Rubio et al. (2009) suggested an effect of milk preservatives on the enzyme reaction of the enzyme-immunoassay they have used to detect Aflatoxin M1 in sheep milk. Klintevall et al. (1991) noticed that milk samples preserved with bronopol had a reduced optical density in a milk ELISA for bovine leukemia virus. High concentrations of bronopol have negatively reduced the assay optical density. In our study, using bronopol as a milk preservative did not have any significant effect on the PAG concentration measured by ELISA. This may be due to the low concentration of bronopol used to preserve our milk samples.

Nickoloff et al. (1984) and Zollner (1993) reported that potassium dichromate may inhibit the enzyme activity and alter the enzyme immunoassay results. Which is seen in the lower correlation between expected and measured PAG content in milk samples in our study. On the other hand, Molina et al. (2009) noticed that milk samples could be preserved with potassium dichromate for 10 days without a significant effect on the ELISA used in their study.

The higher correlation between expected and measured PAG content in UHT milk samples may result from the difficulty of removing the fat content from UHT milk by centrifugation in comparison with the fresh milk, in which the fat removal by centrifugation was more successful. The differences may be also due to action of the proteolytic enzymes in fresh milk,

which may act negatively on the milk PAG content. Some of these enzymes may still active or at least partially active even after pasteurization (Fox and McSweeny, 1998).

4.4.2 Experiment 2: Effect of milk fat, pasteurization, sonication and storage temperature

In the first trial of the second experiment, using untreated full-cream milk in the PAG ELISA induced extremely high recovery rates with very high SD in preliminary studies, so the fat contents of milk samples have to be eliminated. According to Kanungo et al. (2011), milk with higher fat content inhibits the color development in an immunoassay. The effect of milk fat may be due to its role in scattering the light used by the photospectrometer. This scattered light does not follow Lambert-Beer law of absorbance and can result in false positive or false negative results (Datta and Dasgupta, 2010). Skim milk may exert some matrix effect on the immunoassay, owing to its high protein content (Rainard, 2010). This may explain the elevated recovery rates, despite the removal of the milk fat. Higher recovery rates were observed in milk samples with low PAG levels in our study. Similar results were reported by Lee et al. (2003) using a competitive indirect ELISA for detecting deltamethrin in milk. The lower recovery rates in samples stored for 1 or 2 days compared with fresh samples is probably caused by an increased proteolytic activity in UHT skim milk, as have been reported by Lopez-Fandino et al. (1993). Although the removal of the fat contents did not eliminate all factors of disturbance, it is strongly recommended.

In the second trial of the second experiment, when comparing the profiles of the PAG recovery rates during storage within different treatments (pasteurization sonication, and control) at different storage temperatures, the recovery rates were around 100% during the

first 3 days after cooled or frozen storage. The decrease in PAG recovery rates after 5 days may be caused by an increased proteolytic activity in the skim milk as has been reported earlier (Deeth et al., 2002; Janzen et al., 1982). The increase in frozen samples between 3 and 5 days of storage may be caused by dehydration as has been suggested by Allen and Foote (1988) concerning the higher progesterone concentrations in frozen milk samples. Accordingly, milk samples could be kept cooled for 3 days without drastic changes in the PAG content.

The effect of the pasteurization on PAG recovery rates was stronger than the effect of ultrasonic treatment. Cameron et al. (2009) have shown that ultrasound treatment was effective in eliminating the milk spoilage microbes without a negative impact on milk protein components. After heat treatment, PAG concentration was lower regardless of the storage temperature, although the PAG molecules were mostly described as very stable depending on its long half-life of 8 days (Kiracofe et al., 1993). Negative effects of heat treatment on IgG (Chen et al., 1998) or other glycoproteins (Sanchez et al., 1992) in milk samples have been reported. Especially for IgG, it may be related to the unfolding of the molecules at high temperatures (Calmettes et al., 1991) which may alter their immunoreactivity (Dominguez et al., 1997). Since PAG concentration did not differ much after pasteurization, heat treatment can be recommended for milk samples that could not be assayed within 72 hours after collection.

4.5 Conclusions

It can be concluded that fresh and UHT milk were better choices for preparation of control and standard samples used in PAG ELISA. Milk samples stored up to 3 days can be used in the analysis without marked change in their PAG content. When using milk preservatives, bronopol appears to be the most desirable choice. Measurement of PAG in milk is possible

without namable losses during the first 3 days of cooled storage. While doing so, the milk should be defatted to avoid milk fat interference with assay results.

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Chapter 5

Concluding Remarks

The aim of the present dissertation was to examine the reliability of a PAG polyclonal ELISA developed by Friedrich and Holtz (2010) in goats, different cattle breeds and bovine milk. The dissertation is structured in three studies. In the first study PAG levels were determined using three different antisera throughout pregnancy and in the postpartum period in Boer goat does. The second study was to investigate the effect of the breed and production purpose on the PAG levels throughout pregnancy in cattle. The third study was to investigate the effects of milk fat content, milk treatments, milk type and milk preservatives on the PAG ELISA in bovine milk.

The first study addressed the question to what extent plasma PAG determination may serve as a means of early pregnancy detection in goats in a similar way as it is practiced in cows, and whether an ovine or bovine PAG-ELISA may be utilized to this end. Blood samples were collected from eight pregnant pluriparous Boer goat does twice weekly during the first seven weeks and the last four weeks of pregnancy and weekly in-between and during four weeks following parturition. Plasma PAG concentrations (mean ± SEM) were determined using a competitive enzyme-linked immunosorbent assay. Assays were conducted with polyclonal antisera raised in rabbits against purified preparations of caprine (AS#706), ovine (AS#780) and bovine PAG (AS#726). In the assay systems, purified bovine PAG served as standard and tracer and goat anti-rabbit IgG served as coating antibody. With the antibody raised against caprine PAG (AS#706) a steep increase to a climax of 69 ± 9 ng/ml on day 56 of pregnancy was followed by a gradual decline to 16 ± 3 ng/ml at parturition and 0.3 ± 0.07 ng/ml four weeks postpartum. The results achieved with the anti-ovine PAG (AS#780) showed close similarity, a maximum of 92 ± 14 ng/ml being reached at 56 days of pregnancy. With anti-bovine PAG (AS#726), the PAG level increased to a maximum of 3.1 ± 0.2 ng/ml on day 105 of pregnancy and fluctuated around 3 ng/ml until the end of pregnancy. The difference between pregnant and non-pregnant does reached a significant level 21 days after conception,

one week earlier than with caprine and ovine antisera. For future studies, the extraction and characterization of other PAG molecules from other stages of pregnancy and the use of antisera prepared against those new molecules may present a new image of the PAG profile in goats. It will be very interesting to use the bovine antiserum for the PAG determination in sheep and to investigate its effect on sheep PAG profile, and to compare it with ovine and caprine antisera.

The goal of the second study was to compare PAG pregnancy profiles of a dual-purpose (Simmental) and two beef breeds (Uckermark and Aubrac) with the profile of a specialized dairy breed (Holstein-Friesian). The Holstein-Friesian cows were sampled weekly; the levels of the other breeds were presented at three-week intervals. The overall significant breed difference recorded ($P=0.013$) was obtained from deviations in PAG levels of Simmental and Uckermark cows from the Holstein-Friesian and Aubrac breed during the initial three weeks and from 23 weeks of pregnancy onward. During the time between 4 and 22 weeks, when the detection of pregnancy is an issue, PAG levels of the studied breeds were in close agreement ($P>0.05$). No significant effect of body mass of cow or calf (relative to mass of dam) was detected. These findings imply that the PAG pregnancy test may be executed irrespective of breed or type of cow, affirming the suitability of the test as a valuable asset in cattle husbandry. A future study should consider recording other parameters associated with the different breeds such as the placental masses and measurements, metabolic traits of the studied breeds, comparative study of the half-life of the PAG in the different breeds. Investigation of the temporal expression pattern of different PAG isoforms in the studies breeds should be considered. The examination of PAG levels in the first few weeks of pregnancy in beef cattle in which the bull did not join the herd until much later than 60 days postpartum, and compare it with that of dairy cattle in the same time period will be very interesting.

The third study was conducted to investigate the effect of milk preservatives, milk fat and different milk treatments on PAG concentrations in milk measured by PAG ELISA. The effect of milk preservatives, milk type and storage duration was investigated using fresh milk (non-homogenized, non-pasteurized), organic milk (non-homogenized, pasteurized) and ultra-high temperature treated (UHT) milk (homogenized, sterilized) with 3.5 % fat. The milk was skimmed by centrifugation and treated either with bronopol, sodium azide, potassium dichromate or left without treatment as a control. Known PAG concentrations were added 2 days, 1 day and shortly before the assay. The effect of milk fat content was studied using UHT milk with two different fat contents (1.5 and 0.3 %) spiked with known concentrations of PAG. The effects of pasteurization and sonication were tested using pooled milk from dairy cows after about 2 weeks of calving. Following milk skimming, samples were pasteurized at 63°C for 30 minutes, sonicated or left without treatment as a control. Samples were then stored at three different temperatures (room temperature, 4°C and -20°C) for different durations (1, 3, 5 and 7 days). In the first experiment, fresh and UHT milk showed the best correlation coefficients between measured and expected PAG concentrations. All milk samples can be stored up to three days before the PAG analysis without drastic change in the measured PAG content. When milk preservatives were added, bronopol gave the best correlation between expected and measured PAG values. Differences in PAG recovery rates between different fat contents of UHT milk were not statistically significant ($P>0.05$). Recovery rates at lower PAG concentrations were significantly higher ($P<0.05$) than those with higher PAG content and freshly prepared samples were significantly higher ($P<0.05$) than those stored for 1 or 2 days. The effect of ultrasonic treatment was less destructive to milk PAG content compared to pasteurization, although the results in the pasteurization group were more uniform throughout storage time. The cooled milk samples did not show drastic change in the recovery rates in the first 3 days of storage. To determine PAG concentration in milk samples using the PAG ELISA, skimmed fresh or skimmed UHT milk was preferred for

the preparation of assay standards and control. When considering milk preservatives, bronopol was found to be the most desirable. The milk samples could be pasteurized and/or kept cooled for 3 days without noticeable changes in PAG content.

. For further studies on the effects and duration of storage UHT milk should not be used because of significant differences to the fresh milk samples. To proof and clarify the identified general effects of milk fat content, storage duration and milk preservatives more explicitly a study should be performed on a much greater number of milk samples. To substantiate the results, samples should be taken and analyzed immediately after milking from advanced pregnant animals to obtain native PAG in fresh milk. Such a study should definitely consider further characteristics of the milk samples, such as somatic cell count, total protein, urea, and fat contents.

Summary

The aim of the PhD dissertation was to examine the reliability of a PAG polyclonal ELISA developed by Friedrich and Holtz (2010) in goats, different cattle breeds and bovine milk. The dissertation comprised three studies. In the first study, pregnancy-associated glycoprotein (PAG) pattern and pregnancy detection in Boer goats were determined using an ELISA with different antisera. The second study investigated PAG profiles in dairy, dual purpose and beef cows. The effects of milk fat content, milk treatments, milk type and milk preservatives on PAG ELISA in bovine milk was determined in the third study.

The first study addressed the question to what extent plasma PAG determination may serve as a means of early pregnancy detection in goats in a similar way as it is practiced in cows, and whether an ovine or bovine PAG-ELISA may be utilized to this end. Blood samples were collected from eight pregnant pluriparous Boer goat does twice weekly during the first seven weeks and the last four weeks of pregnancy and weekly in-between and during four weeks following parturition. Plasma PAG concentrations (mean ± SEM) were determined using a competitive enzyme-linked immunosorbent assay. Assays were conducted with polyclonal antisera raised in rabbits against purified preparations of caprine (AS#706), ovine (AS#780) and bovine PAG (AS#726). In the assay systems, purified bovine PAG served as standard and tracer and goat anti-rabbit IgG served as coating antibody. With the antibody raised against caprine PAG (AS#706) a steep increase to a climax of 69 ± 9 ng/ml on day 56 of pregnancy was followed by a gradual decline to 16 ± 3 ng/ml at parturition and 0.3 ± 0.07 ng/ml four weeks postpartum. The results achieved with the anti-ovine PAG (AS#780) showed close similarity, a maximum of 92 ± 14 ng/ml being reached at 56 days of pregnancy. With anti-bovine PAG (AS#726), the PAG level increased to a maximum of 3.1 ± 0.2 ng/ml on day 105 of pregnancy and fluctuated around 3 ng/ml until the end of pregnancy. The difference

between pregnant and non-pregnant does reached a significant level 21 days after conception, one week earlier than with caprine and ovine antisera.

In the second study, the PAG pregnancy profiles of a dual-purpose (Simmental) and two beef breeds (Uckermark and Aubrac) were compared with the profile of a specialized dairy breed (Holstein-Friesian). The Holstein-Friesian cows were sampled weekly; the levels of the other breeds were presented at three-week intervals. The overall significant breed difference recorded ($P=0.013$) was obtained from deviations in PAG levels of Simmental and Uckermark cows from the Holstein-Friesian and Aubrac breed during the initial three weeks and from 23 weeks of pregnancy onward. During the time between 4 and 22 weeks, when the detection of pregnancy is an issue, PAG levels of the studied breeds were in close agreement ($P>0.05$). No significant effect of body mass of cow or calf (relative to mass of dam) was detected. These findings imply that the PAG pregnancy test may be executed irrespective of breed or type of cow, affirming the suitability of the test as a valuable asset in cattle husbandry.

The third study was conducted to investigate the effect of milk preservatives, milk fat and different milk treatments on PAG concentrations in milk measured by PAG ELISA. The effect of milk preservatives, milk type and storage duration was investigated using fresh milk (non-homogenized, non-pasteurized), organic milk (non-homogenized, pasteurized) and ultra-high temperature treated (UHT) milk (homogenized, sterilized) with 3.5 % fat. The milk was skimmed by centrifugation and treated either with bronopol, sodium azide, potassium dichromate or left without treatment as a control. Known PAG concentrations were added 2 days, 1 day and shortly before the assay. The effect of milk fat content was studied using UHT milk with two different fat contents (1.5 and 0.3 %) spiked with known concentrations of PAG. The effects of pasteurization and sonication were tested using pooled milk from dairy

cows after about 2 weeks of calving. Following milk skimming, samples were pasteurized at 63°C for 30 minutes, sonicated or left without treatment as a control. Samples were then stored at three different temperatures (room temperature, 4°C and -20°C) for different durations (1, 3, 5 and 7 days). In the first experiment, fresh and UHT milk showed the best correlation coefficients between measured and expected PAG concentrations. All milk samples can be stored up to three days before the PAG analysis without drastic change in the measured PAG content. When milk preservatives were added, bronopol gave the best correlation between expected and measured PAG values. Differences in PAG recovery rates between different fat contents of UHT milk were not statistically significant ($P>0.05$). Recovery rates at lower PAG concentrations were significantly higher ($P<0.05$) than those with higher PAG content and freshly prepared samples were significantly higher ($P<0.05$) than those stored for 1 or 2 days. The effect of ultrasonic treatment was less destructive to milk PAG content compared to pasteurization, although the results in the pasteurization group were more uniform throughout storage time. The cooled milk samples did not show drastic change in the recovery rates in the first 3 days of storage. To determine PAG concentration in milk samples using the PAG ELISA, skimmed fresh or skimmed UHT milk was preferred for the preparation of assay standards and control. When considering milk preservatives, bronopol was found to be the most desirable. The milk samples could be pasteurized and/or kept cooled for 3 days without noticeable changes in PAG content.

Curriculum Vitae

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PERSONAL DATA

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