
An overview on Pregnancy Associated Glycoproteins in Cattle and Buffalo

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Abstract

Accurate pregnancy detection is one of the prime requisites in reproductive management of domestic animals as it indirectly depicts fertility of the animal. Early pregnancy detection in animals is important so as to monitor reproductive losses. Though hormonal profiling of progesterone is an important method of pregnancy detection in domestic species; it usually results in false positivity. Pregnancy marker(s), which is embryo specific and depicts presence and viability of the embryo, should be considered ideal candidate for developing pregnancy diagnostics. Pregnant Associated Glycoproteins (PAGs) are potential biomarker in early pregnancy in cattle and buffalo. They belong to the aspartic proteinase family but proteolytically inactive due to key mutations. In this review, the scope and prospects of pregnancy associated glycoproteins as potential pregnancy biomarkers in cattle and buffalo species, is discussed.

Keywords: Conceptus; Biomarkers; Pregnancy diagnosis; Pregnancy Associated Glycoprotein

Introduction

Optimum reproductive efficiency in domestic species is obtained by better conception rate which in turn is affected by the series of successful interactions between the conceptus and maternal reproductive tract during pregnancy. These interactions between the conceptus and maternal system are important both for maternal recognition of pregnancy as well as embryonic development by blocking PGF2 α secretion from the uterus, sustaining the developing conceptus (Spencer *et al.*, 2004). Therefore, establishment of pregnancy involves synchronous interactions between two interdependent systems i.e. the conceptus (embryo and extra embryonic membranes) and the maternal system. The synchrony between these systems during the peri-attachment period is influenced by potential signals from the conceptus which facilitate in recognition of pregnancy.

These signals from conceptus to the maternal unit are crucial because they interrupt normal cyclic luteal regression thereby enhancing embry-

onic survival and sustenance of pregnancy. The important signals to maternal system to sustain pregnancy are mainly proteins or steroids (Sousa *et al.*, 2006, Szafranska *et al.*, 2006, Roberts *et al.*, 2007). These signals can be considered as potential markers of embryonic viability as they influence pregnancy recognition and successful implantation. Pregnancy associated glycoprotein is one such bimolecular secreted during pregnancy and possess functions viz. embryo protective, placentogenesis, placental remodeling and maternal recognition of pregnancy and sustenance of pregnancy. This review throws some light on pregnancy associated glycoprotein, its genomic characteristics and physiological functions in cattle and buffaloes.

Pregnant Associated Glycoproteins (PAGs)

Pregnant associated glycoproteins comprise a large group of placental Aspartic Proteinase superfamily that is expressed in pre-placental trophoblast (TR) and after implantation in the trophoctoderm (TRD) – the chorionic epithelium of eutherian mammals with different placenta types. The first member of the family was isolated by Butler *et al.* (1982) from bovine placenta and named as pregnancy specific protein B (PSPB). Subsequently, re-

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Table 1. Native PAG protein isolated from placental tissues of different species

Name	Molecular weight (kDa)	pH	Day of pregnancy	Species	Reference
bubPAG	52-77	-	Mid-late pregnancy	Buffalo	Barbato <i>et al.</i> (2008), Singh <i>et al.</i> (2005)
bPAG-1	67	4.4-5.4	90	Cattle	Xie <i>et al.</i> (1991), Zoli <i>et al.</i> (1991), Butler <i>et al.</i> (1982)
zebuPAG-1	51-69	4.4-6.7	70	Zebu cattle	Sousa <i>et al.</i> (2002)
oPAG-1	47-90	4.1-5.9	15-25	Sheep	Willard <i>et al.</i> (1995)
cPAG-1	62,59,55	4.9-6.2	48-69	Goat	Garbayo <i>et al.</i> (1998)
pPAG-1	42-79	-	15-77	Pig	Szafranska <i>et al.</i> (1995)
ePAG	37,41	4.8-6.2	15	Horse	Green <i>et al.</i> (1999)
EbPAG	50-71	3.7-7.4	45-120	European bison	Kiewisz <i>et al.</i> (2008)
AmPAG	56-75	4.4.6	120	American bison	Kiewisz <i>et al.</i> (2008)

lated proteins have been identified with different names such as PAG-1 (Xie *et al.*, 1991, Zoli *et al.*, 1991), PSP-60 (Mialon *et al.*, 1993), SBU3 (Atkinson *et al.*, 1993) in ruminant ungulates. These proteins are detectable in maternal blood and are reported to be useful as prenatal markers for pregnancy diagnosis in various domestic and wild ruminants. PAGs were isolated using biochemical procedures from cotyledons in cattle (Zoli *et al.*, 1991, Sousa *et al.*, 2002, Klisch *et al.*, 2005), ewe (Xie *et al.*, 1997, El Amiri *et al.*, 2003, 2004), goat (Garbayo *et al.*, 1998), buffalo (Barbato *et al.*, 2003, 2008; Singh *et al.*, 2005) and bison (Kiewisz *et al.*, 2008). Purified and semi-purified preparations were used to immunized rabbits and the antisera has been used to develop homologous (Sasser *et al.*, 1986; Humblot *et al.*, 1988; Zoli *et al.*, 1992; Mialon *et al.*, 1993, 1994, El Amiri *et al.*, 2006, 2007) and heterologous radioimmunoassay (RIA) systems (Ranilla *et al.*, 1994, Gonzalez *et al.*, 1999, Ayad *et al.*, 2007) as well as enzyme linked immunosorbent assay (ELISA) systems (Green *et al.*, 2005). Apparent molecular mass of purified PAG in cattle ranges between 37 to 78 kDa. The variable degree of glycosylation in different PAG plays important role in regulating the half life of the PAG proteins. There are various PAGs (PAG-1 to PAG-22) secreted from placenta at different stages of pregnancy. The first PAG purified from bovine placenta is known as boPAG-1 (Xie *et al.*, 1991, Zoli *et al.*, 1991). It has molecular weight of 67 kDa and has various isoforms (pI 4.4, 4.6, 5.2 and 5.4). In bovine, PAG-1, -3, -4, -5, -6, -7 and 9 are expressed

in binucleate cells (BNC), while boPAG-2, -8, -10, -13 are expressed in both mononucleate cells (MNC) and binucleate cells (Green *et al.*, 2000). Other PAGs isolated from bovine cotyledons are boPAG 56kDa, boPAG 67 kDa and boPAG 75 kDa (Klisch *et al.*, 2005). Bovine PAG-1 polypeptides showed 73 % amino acids identity with ovine PAG1 exhibiting interspecies conservation of PAG-1.

The NH2 terminus of boPAG beginning with Arg-34 suggests further proteolytic processing even after the cleavage of signal peptide. Critical amino acids substitution at the active site of bovine and ovine PAG-1 suggests that the proteins are enzymatically inactive as many possess nucleotide substitutions within the catalytic center (Xie *et al.*, 1997). Comparing PAG sequences both within a single species and between species reveals segments of primary structure that are hypervariable and others that are relatively constant. The first 15 amino acids of each PAG molecule which appear to constitute the signal peptide, amino and the carboxyl termini are highly conserved apart from other constant regions. Further a conserved site for EcoR1 is present in every binucleate cell-specific PAG (Garbayo *et al.*, 2008). The conserved regions are ones that are internal and structurally important for retaining the overall three dimensional fold of the molecule (Guruprasad *et al.*, 1996). A conserved motif for propeptide removal is the sequence ISF↓RGS, present in all members of the PAG-1 group in contrast to the members of the PAG-2 group, where the site of propeptide removal

is difficult to ascertain

Genomic characteristics of Pregnancy Associated Glycoproteins

The PAGs are a multigene family expressed in placenta of eutherian mammals and their expression varies spatially as well as temporally during gestation (Garbayo *et al.*, 2008). Recent investigations have demonstrated that different PAG cDNAs are not expressed coordinately throughout pregnancy. Some are expressed early, while others only when pregnancy progresses (Garbayo *et al.*, 2000, Green *et al.*, 2000, Hughes *et al.*, 2000). Screening of placental libraries with nucleic acid probes has identified more than 100 cDNAs that are very abundant and code for polypeptides related but generally distinct from PAGs isolated by biochemical procedure. Multiple PAG genes have been cloned and identified in domestic and wild ruminants as well as in pigs (Xie *et al.*, 1991, Szafranska *et al.*, 1995; Garbayo *et al.*, 2000, Vawter *et al.*, 2004).

These identified cDNA sequences represent distinct transcripts encoding unique polypeptide PAG precursors. However, the cDNA of PAGs have yet to be cloned in many species including buffalo. Phylogenetic analyses of PAG and PAG like genes indicate that the PAG family originates from an ancient PAG-like precursor (pro-gene) by duplication and positive selection approximately 86 million years ago (Roberts *et al.*, 1996, Hughes *et al.*, 2000, 2003), which is consistent with estimates for the

split of the artiodactyls from other ungulate orders. The conservation and homology of the PAGs in many eutherians (artiodactyla, perissodactyla, carnivora and rodentia) is suggestive of their importance in the reproductive physiology of ungulate species. Cloning of the PAG and PAG- like gene family revealed distinct chorionic transcripts. The number of the PAG mRNA is known to vary between species and their expression can sometimes change during the course of pregnancy. Presumably, the distinct number and pregnancy-stage dependent expression of the PAG transcripts can be associated with specific requirements of fetal development in different species. The PAG family has several conserved regions and shares nearly 50% sequence identity with the Pepsinogen family. PAG genes have been cloned and sequenced in many species such as cattle, sheep (Xie *et al.*, 1991), goat (Garbayo *et al.*, 2000), pig (Szafranska *et al.* 1995) and white tailed deer (Vawter *et al.*, 2004).

PAG-1 gene

PAG-1 gene was first cloned in cattle followed by sheep, goat and pig. Expression of bPAG-1 was detected by RPA (Ribonuclease Protection Assay) as well as Northern hybridization and it was found that mRNA of the bovine, caprine, ovine and porcine PAG genes are detectable from 15-18 days post-coitum or even before (Xie *et al.*, 1991, 1994, 1995, Szafranska *et al.*, 1995, Garbayo *et al.*, 2000; Green *et al.*, 2000, Ushizawa *et al.*, 2005).

Table 2. Cloned cDNA of the PAG-1 gene in various species

Name	Accession number	Amino acids	Day of pregnancy	Species	Reference
Buffalo PAG-1	EU815059	380	30	Buffalo	Jerome <i>et al.</i> (2011)
Buffalo PAG-2	HM468470	367	-		Green <i>et al.</i> (2010)
bPAG-1	M73962 NM17441	380	260	Cattle	Xie <i>et al.</i> (1991)
oPAG-1	M73961	382	100	Sheep	
cPAG-1	AF191326	341	45-115	Goat	Garbayo <i>et al.</i> (2000)
pPAG-1	L34360	389	13-17	Pig	Szafranska <i>et al.</i> (1995)
ePAG	L38511	388	25	Horse	Green <i>et al.</i> (1999)
zPAG	AF036952	388	Term placenta	Zebra	
wtdPAG-1	AY509865	380	85-90	White tailed deer	Brandt <i>et al.</i> (2007)

Structure of the gene

The identification of the structural organization of the PAG genes or their promoters was performed on the basis of identified gDNA sequences and required single gene isolation by genomic bovine or porcine library screenings (~1.2 kbp promoter and ~ 8 kbp gene). The entire exon-intron structures were identified for two PAG genes only, bovine PAG-1 and porcine PAG-2. The gDNA sequencing of the PAG genes and comparison of the ORF of previously cloned cDNAs allowed structural identification of 9 exons and 8 introns (A-H) in the bovine and porcine PAG genes. In bovine PAG-1 gene, the exons are 99-200 bp long and introns ranges from 85 bp up to ~1.8 kbp. Exon 1 codes for signal peptides and propieces, exons 2-5 code for the N-terminal lobe and exons 6-9 code for the C-terminal lobe of the mature polypeptide (Xie *et al.*, 1995). Functional activity of PAG genes begins with the cloning and sequencing of the promoter. So far, only a few promoter sequences of the PAG genes have been identified and deposited in GenBank. The comparison of the PAG promoter sequences revealed that their proximal regions are similar to each other, but not to promoters of other aspartic proteinase or other trophoblast-expressed genes.

A 20 kb genomic fragment, which contained the whole bPAG-1 gene, was isolated from the λ GEM-11 genomic libraries (Clone bpg 2000). The transcription start point (tsp) is located 53 or 54 bp upstream from the start codon (ATG) and 19 bp downstream from a 5'-TATATAA sequence. The exon 1 has both noncoding (52 bp) and coding (53 bp) sequence. The latter provides the entire 15 aa

signal peptide region and an additional 8 bp encoding for the beginning of the pro-peptide. Exon 2 encodes the remainder of the pro-peptide (aa 1-38) (Xie *et al.*, 1995). The mature portion of the protein is coded by the remainder of exon 2, exons 3 to 8 and the first 150 bp of exon 9. A total of 355 bp after the stop codon (TAA) includes a poly-adenylation signal (AATAAA), which is located at the identical position within the bPAG-1 cDNA. The organization of PAG-1 gene is similar to that of all other known aspartic proteinase encoding genes.

Variant of boPAG-1 gene

Placental PAG-1 cDNA was immunoscreened with an anti-bPAG-1 antiserum (Zoli *et al.*, 1991). Out of ten immunopositive clones initially purified and partially sequenced, nine matched clone bp314, the cDNA that encodes bPAG-1. However, one clone (bp 111) was distinctly different from the others. The polypeptide encoded by bp111 cDNA has been named bPAG-1var. The two cDNA, bp 111 and bp 314 showed 91.5 and 86.5 % identities at the nucleotide and amino acid sequences, respectively. The mismatches between bPAG-1 and bPAG var are scattered throughout the lengths of the polypeptide, notably aa 76 in bPAGvar is Gly rather than Ala i.e. the difference likely to have inactivated bPAG-1 as a proteinase is not evident in bPAG-1var (Xie *et al.*, 1995).

Expression of PAGS during pregnancy

Molecular biology investigations deduced that during certain stages of pregnancy some PAGs were expressed, while others were absent (Garbayo *et al.*

Table 3. Recognized structural organization of boPAG-1 gene deposited in GenBank

Accession number	gDNA sequences	Identified by
L27833	EXONS 1-6	Xie <i>et al.</i> (1995)
L22834	EXONS 7-8	
L27832	EXON 9	

Table 4. Identified promoter sequence of PAG-1 gene

Accession number	Length of promoter (bp)	Identified by
L27833	1260	Xie <i>et al.</i> , (1995)

Table 5. Cloned cDNA of the PAG gene family of cattle deposited in the GenBank database.

Name	Accession Number	Coded polypeptide (aa)	Transcripts (Day of pregnancy)	Identified by
boPAG-1	M73962	380	260	Xie <i>et al.</i> (1991, 1994, 1997)
boPAG-2	NM176614.1	376		
boPAG-3	L06153	381		
boPAG-4	AF020506	380	19-25	
boPAG-5	AF020507	377		
boPAG-6	AF020508	379		
boPAG-7	AF020509	341		
boPAG-8	AF0205010	346		
boPAG-9	AF0205011	381		
boPAG-10	AF0205012	380		
boPAG-11	AF0205013	376		
boPAG-12	AF0205014	375		
boPAG-13	AF192330	370-381	19	Green <i>et al.</i> (2000)
boPAG-14	AF192331			
boPAG-15	AF192332			
boPAG-16	AF192333			
boPAG-17	AF192334			
boPAG-18	AF192335			
boPAG-19	AF192336			
boPAG-20	AF192337			
boPAG-21	AF192338			

1999, 2000, Green *et al.*, 2000). The PAG molecules which were predominantly expressed in the binucleate cells (like boPAG-1, -6, and -7) were expressed weakly or not in the day 25 placenta, but they were present at the middle and the end of pregnancy. Others like boPAG-4, -5, and -9 were expressed at day 25 and at earlier stages.

By the use of cDNA microarray analysis Ushizawa *et al.* (2005) demonstrated that several PAG molecules are expressed as early as day 7 to 14 of pregnancy boPAG -11,-16 and -17), days 14 to 21 boPAG -1,-5 to -7,-9 to -13,-15 to -17,-19,-21) or even before (at day 7: boPAG -4,-5 and -6. In species with epitheliochorial placenta, PAGs (eqPAG-1, poPAG-1, and poPAG-2) were reported to be expressed throughout the chorion (Szafranska *et al.*, 1995, Green *et al.*, 1999).

Functions of PAG

The function of PAG family may be complex and combine with specific expression during pregnancy

stages. Some PAG members affect maternal recognition of pregnancy, adhesion of trophoblast and implantation, while other PAG members may be involved in remodeling of the feto-maternal unit during placenta development in various mammalian species. High level of expression in early gestation shows that PAG may be involved in implantation, maternal recognition of pregnancy, placentogenesis and placental modeling. PAG has also been implicated to have luteotrophic, luteoprotective and regarded as a good indicator of embryo viability (Szafranska *et al.*, 2007). PAG proteins were hypothesized as local immunosuppressive effect that can be involved in the maintenance of the histoincompatible feto-maternal unit (Dosogne *et al.*, 2000). The concentration of PAG in maternal circulation depends on the number of the well being embryos/fetuses, higher in twin bearing females (Mialon *et al.*, 1993, Willard *et al.*, 1995, Batalha *et al.*, 2001). The secretion of PAG also differs based on the sex and the breed of the fetus (Zoli *et al.*, 1992).

Potential role of PAG as pregnancy diagnostic marker

Purified native or recombinant PAG proteins and specific anti-PAG sera has led to the establishment of several diagnostic tests for early pregnancy diagnosis based on the detection of PAGs in maternal blood plasma or serum and provide an alternative method for determining the embryonic survival (Szenci *et al.*, 1998, 2003). The major significance of PAG assays in ruminants, similar to urine or plasma hCG tests in primates, done by RIA and ELISA system is the direct identification of a placental product present in the maternal system that can be used as a marker of a viable pregnancy and embryonic mortality in various ruminants (Zoli *et al.*, 1992, Humblot, 2001, Szenci *et al.*, 2003, Gonzalez *et al.*, 2004). These glycoproteins could be detected in the maternal circulation at around the time when the trophoblast forms definitive attachment to the uterine wall (Sasser *et al.*, 1986; Zoli *et al.*, 1992). The measurement of PAG protein concentrations in maternal blood or milk of ruminants allow for 76.6 -100% accuracy rates for early pregnancy diagnosis (Gonzalez *et al.*, 2001, 2004). The mean concentration of PAG in pregnant cattle is between 0.5-0.8 ng/ml. The half-life of PAG was estimated to be 7.4 to 9 days (Kiracofe *et al.*, 1993; Mialon *et al.*, 1993; Ali *et al.*, 1997). PAG has been reported to be pregnancy stage dependent; it increases as pregnancy progresses (Green *et al.*, 2005). The homologous and heterologous PAG assays allow for early pregnancy diagnoses in domestic cattle (Sasser *et al.*, 1986, El Amiri *et al.*, 2000, Humblot, 2001, Green *et al.*, 2005), sheep (Ranilla *et al.*, 1994, Gajewski *et al.*, 1999), goats (Gonzalez *et al.*, 1999), white tailed deer (Osborn *et al.*, 1996) and other wild ruminants (Huang *et al.*, 1999). Heterologous RIA systems such as RIA 706 and RIA 708 have been developed by Perenyi *et al.* (2002) with antisera raised against PAG molecules isolated from caprine placenta. These RIA systems have been found to be more sensitive and specific than homologous RIA. Sandwich ELISA has been developed between day 24 and 28 of pregnancy by using semi purified PAG proteins (Green *et al.*, 2000). Bovine PAG RIA as compared to other methods of early pregnancy diagnosis has been reported to be more sensitive as well as specific (Skinner *et al.*, 1996). A commercial ELISA kit using purified PAG-1 has been developed with the

name BioPRYN by Biotracking Company (USA). It has been found successful in detecting pregnancy by day 28 post-insemination having a success rate of 97%.

Research on PAG in buffaloes

Attempts have been made to isolate and purify pregnancy associated glycoproteins in buffalo but only partial purification could be achieved (Barbato *et al.*, 2003, Singh *et al.*, 2005). Singh *et al.* (2005) isolated six proteins from buffalo placental extract with molecular weights of 78, 67, 53, 42, 33 and 26 kDa. Dot ELISA developed to diagnose pregnancy by using hyper immune sera against these partially purified proteins showed a success rate of 68%. Protein sequence with the name of PAG75_BUBBU (P85048) has been submitted by Barbato *et al.* (2006). Recently, Barbato *et al.* (2008) isolated and purified distinct buffalo PAG proteins by Vicia villosa agarose affinity chromatography. Western blotting with anti-PAG sera showed that the apparent molecular masses of the immunoreactive bands from the Vicia villosa agarose peaks range from 59.5 to 75.8 kDa and from 57.8 to 73.3 kDa from the midpregnancy and late pregnancy placentas, respectively. Amino-terminal microsequencing of these immunoreactive proteins has allowed the identification of three distinct buffalo PAG sequences, which have been deposited in the SwissProt database as RGSXLTIHPLRNIRDDFFYVG (Acc. no. P85048), RGSXLTIPLRNIID (Acc. no. P85049) and RGSXLTHLPLRNI (Acc. no. P85050). Karen *et al.* (2007) studied PAG concentration using heterologous double antibody RIA for diagnosis of pregnancy in buffalo between days 19 and 55 post-breeding. The sensitivity of PAG-RIA test was 11.1% at days 19-24 and reached 100% from day 31 after breeding. The specificity of test ranged from 90 to 100% from 19 to 55 days post-breeding, indicating PAG-RIA test as highly accurate for detecting pregnancy in buffaloes from day 31 onwards after breeding. Genomic study on buffalo PAG carried by Jerome *et al.* 2011 revealed buffalo PAG-1 gene possess conserved regions and key mutations similar to bovine rendering them proteolytically inactive. It is evident that expression of buffalo PAG-1 gene was throughout the pregnancy starting from day 30 to term (Jerome *et al.* 2011b). Molecular modeling studies of buffalo PAG-1 re-

vealed its bilobed structure with pepstatin binding clefts near the active region (Jerome et al. 2011c).

Conclusion

In conclusion, optimum reproductive management in domestic animals is possible by accurate pregnancy diagnosis which in turn depicts animals, fertility. With the advent of 'omics' research viz. genomic, proteomic, transcriptomics such endeavors will result in the development of novel pregnancy biomarkers and their diagnostics.

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