

# The Pregnancy Diagnosis in Buffalo Species: Laboratory Methods

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**Abstract:** Pregnancy diagnosis plays an important role in the reproduction management of ruminants since embryonic mortality has a substantial impact on the fertility of a herd. Most of the embryonic losses occur during the first days after fertilization and during the process of implantation. So it is very important to discriminate, with an early pregnancy diagnosis, non-pregnant from pregnant animals. Hormone analysis to detect pregnancy may be utilized as a more simple technique as an alternative of rectal palpation or ultrasound. In the last years, a large polymorphic family of placenta-expressed proteins has been discovered in ruminant species and used for pregnancy diagnosis. Members of this family are named pregnancy-associated glycoproteins (PAG), being synthesized in the mono- and binucleate cells of the ruminant's trophoblast. Part of them is released in the maternal blood circulation where they can be assayed by different laboratory techniques. Due to large variety of expressed molecules and to large variations in the post-translational processing of the PAG, different immuno-systems present different ability to quantify the PAG released in blood. The assay of PAG can also bring very interesting information for researchers working in programs focused on the study of embryonic and fetal mortalities, as well as on embryo biotechnology, animal nutrition or infectious diseases resulting in pathologies affecting the pregnancy.

**Keywords:** Pregnancy marker, hormone, progesterone, estrone sulfate, PAG, embryonic mortality.

## INTRODUCTION

The world buffalo population is increasing continuously and was estimated to be more than 194 million in 2010 as reported by FAO (FAOSTAT homepage). More than 95 percent of the world population is found in Asia where buffalo play a leading role in rural livestock production. Over the last decades buffalo farming has widely expanded in Mediterranean areas and in Latin American countries and several herds have also been expanded in Central and Northern Europe. It is clear that buffaloes play a prominent role in rural livestock production.

Reproductive efficiency is the primary factor affecting productivity. Accurate diagnosis of pregnancy or non-pregnancy and re-enlistment of non pregnant buffalo into an appropriate breeding protocol, are essential component of successful reproductive programs. Various methods aimed at improving detection of pregnancy have been developed.

Non return to oestrus and rectal palpation of reproductive organs have been the common methods adopted for pregnancy diagnosis in cow. Oestrus behaviour in buffalo has a lower intensity than in cows and is therefore much more difficult to detect by observation, thus non return to oestrus is misleading as buffaloes remain silent without being pregnant [1].

Therefore, the probability of misdiagnosis of pregnant females by oestrus observation appears to be increased. This may be confounded by a small proportion of pregnant buffaloes expressing oestrus [2].

Transrectal palpation is considered to be an accurate method of pregnancy diagnosis only from day 30-45 post mating [3]. A few studies point out that the procedure may increase the risk of iatrogenic embryonic mortality [4]. The procedure however, does not provide any information about the viability of the embryo/fetus during stages of pregnancy [5]. Compared to cattle rectal palpation in buffaloes must be gentle as the rectal mucosa is more fragile and bleed easily.

Transrectal ultrasonography diagnosis in buffalo can be adopted successfully from Day 28-30 after service [6]. At Day 30 it is possible to observe the fetal heart beats [1]. The sensitivity reaching 100% from Day 31 after mating onward [3, 7]. The main advantage of the ultrasound scanning is that it can give an accurate diagnosis earlier than rectal palpation, exceeding palpation in the amount of information obtained from each animal. Anyhow, it is necessary to restrain the animals and have a proficient operator.

## LABORATORY METHODS FOR PREGNANCY DIAGNOSIS

Different laboratory tests have been developed and utilize reproductive hormones as indicators of presence of a viable pregnancy. However the research to

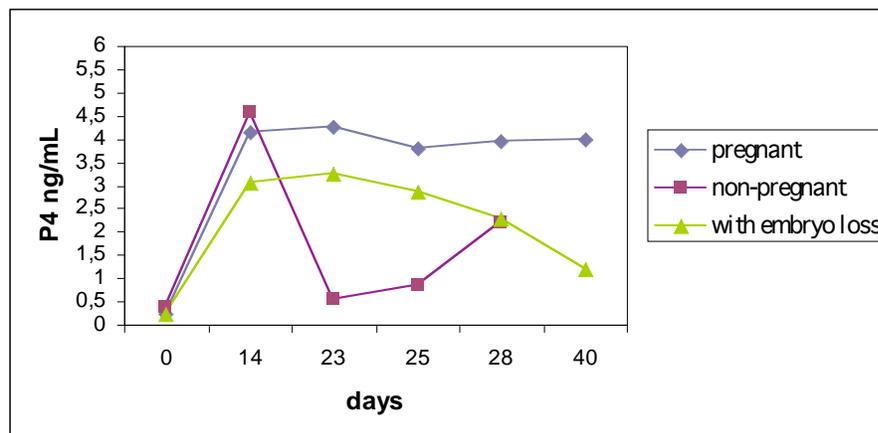
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develop tests continues because these methods are non invasive and alternative of rectal palpation or ultrasound.

### PROGESTERONE HORMONE

Concentration of progesterone in blood at 20 and 24 days post breeding has been used as a tool for early pregnancy diagnosis [8]. However, the accuracy of predicting pregnancy on the basis of high blood progesterone levels at 21 days was only 66.7 % [9] or 75% [10]. In general, a single progesterone analysis does not provide sufficient information to evaluate the pregnancy status accurately [11]. The accuracy of positive pregnancy diagnosis using milk progesterone has been documented for buffalo [12]. Kaul and Prakash [13] accurately diagnosed pregnancies in 57.1%, 69.5% and 75 % of buffaloes respectively on days 20, 22 and 24 post insemination. However, milk sample are not available from non-lactating animals [11]. Anyhow, the technique based on progesterone measurement may be considered as a highly accurate test for non-pregnancy instead of pregnancy, as the concentration of progesterone reflects the function of the corpus luteum and not the presence of an embryo or foetus, so animals that undergoing extended cycle for any reason may be diagnosed positive. The factors which may contribute to misclassification are irregular cycles, early embryonic death, which varies from 19 to 25 days in buffalo, the uterine pathology resulting in persistence of corpus luteum, luteal cysts, incorrect timing of insemination and embryonic death occurred after day 24.

An example of buffalo plasma progesterone trend in different reproductive status is shown in Figure 1 (Barbato and Barile, unpublished data).



**Figure 1:** Trend in serum progesterone (P4) related to pregnancy outcome in buffalo cows artificially inseminated (day 0=AI).

### ESTRONE SULPHATE

The estrone sulfate is produced by the fetomaternal axis or the conceptus and therefore its presence in urine, milk, feces or blood is an indicator of pregnancy.

The appropriate day at which estrone sulfate detection is possible, in buffalo species, is at day 150 of gestation in the serum [14, 15]. A positive test indicates a viable fetus. Hung and Prakash [16] recorded a progressive increase in estrone sulfate concentrations in buffalo plasma after the 4<sup>th</sup> or 5<sup>th</sup> month of pregnancy. Therefore, in the buffalo, the pregnancy diagnosis by using the estrone sulfate assay is tardive. A negative result can express a state of not pregnancy but it does not exclude the beginning of a pregnancy. Rather, this assay allows to assure the fetal vitality in the last two bystanders of pregnancy.

So, it seems clear that in this species is particularly useful a reliable and accurate method for early detection of pregnancy.

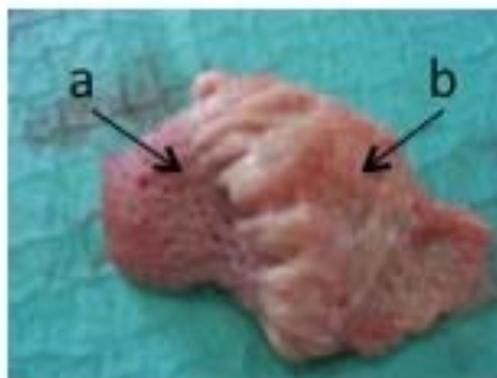
To find a non-invasive, reliable and more practical technique of early pregnancy diagnosis, overcoming the problems associated with progesterone assay, researchers have studied a method to analyse proteins secreted by placental membranes and detectable in maternal circulation.

### PREGNANCY-ASSOCIATED GLYCOPROTEINS

Pregnancy-associated glycoprotein (also called pregnancy-specific protein B, pregnancy specific protein 60 and SBU-3 antigen) constitute a large family of glycoproteins expressed in the outer epithelial cell layer of the placenta of Eutherian species. They are synthesized by mono and binucleate trophoblastic



**Figure 2:** A 14 weeks buffalo fetus with visible placentomes (button-shaped structures) characteristic of ruminant placentation.



**Figure 3:** Single placentome entire (left) and in the single structure of caruncles (a) and cotyledons (b). From the cotyledons tissue the PAGs are isolated.

cells, some of them being secreted in maternal blood from the moment when the conceptus becomes more closely attached to the uterine wall and formation of placentomes begins (Figures 2 and 3) [17].

The PAG (Pregnancy-associated glycoprotein) family were isolated from cotyledons of cow [18-20], ewe [21, 22], goat [23], buffalo [24], bison [25], moose and elk [26]. PAG belong to the aspartic proteinase gene family [27]. However, most PAG molecules are assumed to be enzymatically inactive due to key mutations within their binding cleft [28]. On the basis of molecular biological data, it is estimated that cattle, sheep and probably all ruminants possess many, possibly 100 or more, PAG genes [29, 30]. In bovine, 22 boPAG genes (boPAG-1 to boPAG-22) were cloned and fully sequenced. The number of PAG gene is lower in ovine (15 genes) [21, 29] and caprine species (about 11 genes) [31]. Several bovine, ovine, caprine and

buffalo closely related PAG molecules (63–87% N-terminal amino acid identities) have been made available and have been used to produce antisera for radioimmunoassay (RIA) development [32].

The measurement of circulating concentrations of PAG as a biochemical marker of pregnancy in various ruminant species is established [33-38]. Recently different chromatography allowed identification of new PAG from buffalo placenta (Figure 4) [24]. At the same time Carvalho *et al.* [38] identified PAG immunoreactivity in granules in BNC (binucleate cell) from buffalo.

The PAG concentrations in buffalo species were determined by using heterologous PAG RIA systems [3, 39].

Karen *et al.* [3] studied PAG concentration using heterologous double antibody RIA for diagnosis of

Protein	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
wbPAG <sub>62kDa</sub> *	R	G	S	X	L	T	H	L	P	L	R	N	I							
wbPAG <sub>68kDa</sub> *	R	G	S	X	L	T	H	L	P	L	R	N	I							
wbPAG <sub>70kDa</sub> *	R	G	S	X	L	T	I	L	P	L	R	N	I	I	D					
wbPAG <sub>73kDa</sub> **	R	G	S	X	L	T	I	H	P	L	R	N	I							
wbPAG <sub>75kDa</sub> *	R	G	S	X	L	T	I	H	P	L	R	N	I	R	D	F	F	Y	V	G
Consensus	R	G	S	X	L	T	-	-	P	L	R	N	I	-	D					

**Figure 4:** Microsequence analysis of the buffalo PAG. \*Indicates mid-pregnancy origin of the buffalo placenta. \*\*Indicates late-pregnancy origin of the buffalo placenta [24].

pregnancy in buffalo between days 19 to 55 post-breeding. This study has suggested the PAG-RIA test as highly accurate for detecting pregnancy in buffaloes from day 31 onwards after breeding.

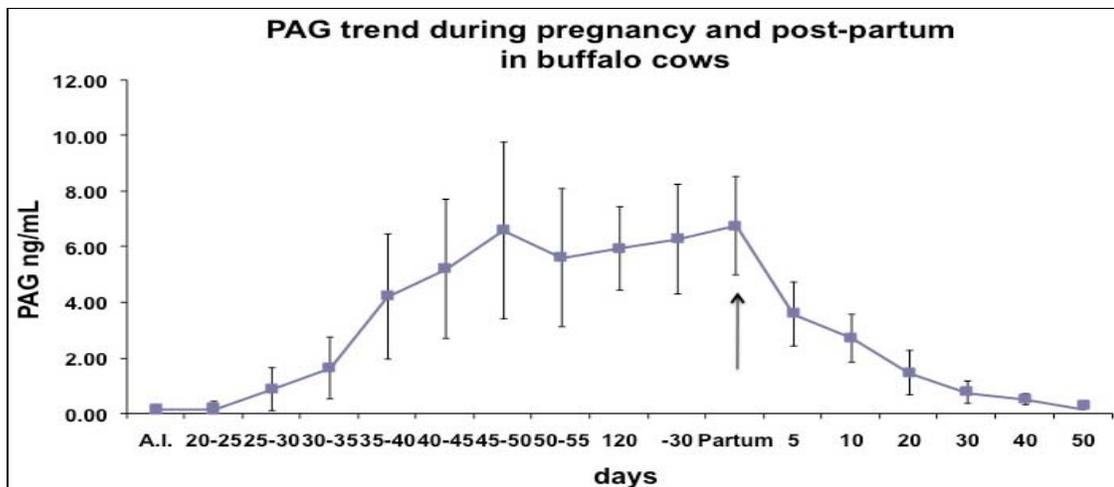
In a study of Barbato *et al.* [39], concentrations of pregnancy-associated glycoproteins (PAG) were determined in buffalo cows (*Bubalus bubalis*) by using three different RIA systems (RIA-497, RIA-706 and RIA-708). Blood samples were collected from Week 0 until Week 28 of pregnancy, and from parturition until Week 10 postpartum. During pregnancy, concentrations of PAG were detectable at Week 6 by the use of the three above-mentioned RIA systems ( $3.9 \pm 1.3$  ng/mL,  $9.7 \pm 1.3$  ng/mL and  $9.9 \pm 0.7$  ng/mL, RIA-497, RIA-706 and RIA-708, respectively). Concentrations increased gradually until Week 28, reaching  $39.6 \pm 4.0$  ng/mL (RIA-497),  $50.5 \pm 11.9$  ng/mL (RIA-706) and  $68.2 \pm 20.8$  ng/mL (RIA-708). Over the whole gestation period, PAG concentrations determined by RIA-706 and RIA-708 were strongly correlated, RIA-708 giving the higher concentrations. At parturition, mean concentrations ranged from  $34.9 \pm 4.0$  (RIA-497) to  $84.7 \pm 10.6$  ng/mL (RIA-708). Thereafter concentrations decreased rapidly, reaching

very low levels (< 1.0 ng/mL) at Week 8 postpartum (Figure 5). So, PAG concentrations measured by three RIA systems showed distinct profiles from those previously described in bovine species, with higher concentrations measured by RIA-706 and RIA-708 at Week 6 after artificial insemination, and lower peripartum levels. These results suggest that the buffalo pregnancy proteins are better recognized by the antisera raised against the caprine PAG. Interestingly, by using these systems, concentration of PAG were remarkably distinct from those measured in cattle, reaching higher levels at the 6th week of pregnancy and low levels postpartum.

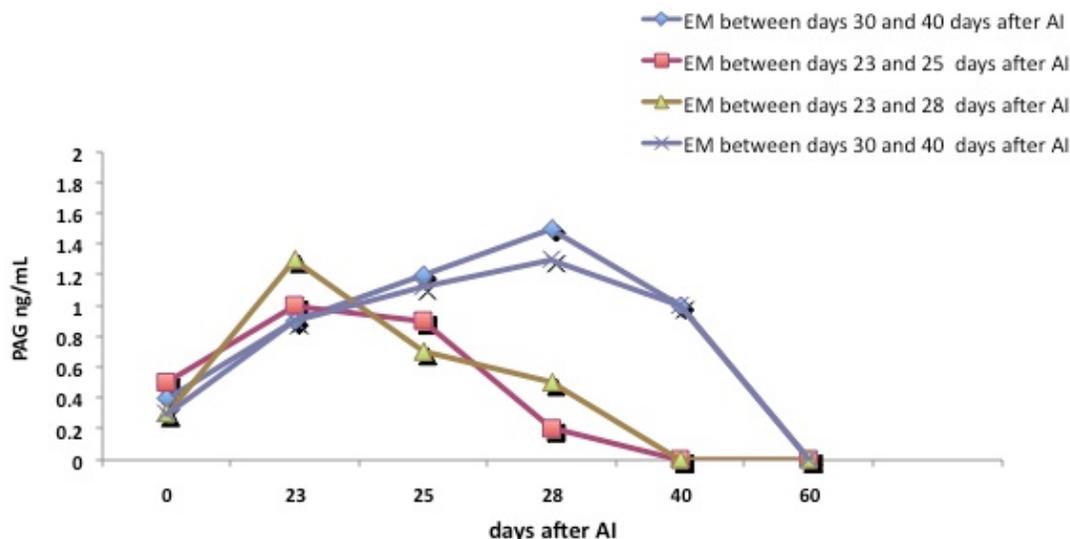
The use of PAG assay is also useful throughout the gestational period in order to reveal incorrect diagnosis of pregnancy and embryonic mortality (Figure 6) (Barbato and Barile, unpublished data).

**CONCLUSIONS**

Reproductive efficiency is the primary factor affecting productivity. Accurate diagnosis of pregnancy or non-pregnancy and re-enlistment of non pregnant animals into an appropriate breeding protocol, are



**Figure 5:** PAG profile during buffalo pregnancy and post-partum.



**Figure 6:** Plasmatic PAG profile of 4 buffalo cows in which embryonic mortality had been diagnosed using ultrasound examination.

essential component of successful reproductive programs.

In practice, the measurement of PAG concentrations in peripheral maternal circulation has been used for both pregnancy confirmation and the follow-up of the trophoblastic function. The first aspect can help veterinarians and breeders in the management of reproduction, while the second represents a powerful tool for investigators involved in studying factors affecting embryo and fetal mortality and embryo biotechnology.

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