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Enzyme link immuno assay for early detection of pregnancy associated glycoprotein's in African dwarf goat

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ABSTRACT: A pregnancy test based on detection of the goat pregnancy associated glycoprotein (caPAG) by the ELISA method was designed and carried out on serum from slaughtered goats. The objective of the study was to describe the ability of this test to detect pregnancy associated glycoprotein and also their ability to discriminate between pregnant and non-pregnant females of African dwarf goats. The test was evaluated and compared to a post-mortem clinical pregnancy examination.
In order to perform the test, rabbits were immunized with caPAG initially purified from cotyledons collected from pregnant goat. The antibodies obtained were biotinylated, titrated, and used in a "Sandwich" ELISA to detect caPAG in goats sera. Blood samples as well as informations related to each animal (pregnancy status, gestational stage, presence of corpus luteum or milk) were collected on 506 slaughtered goats, and the sera obtained were tested for the presence of caPAG.
The ELISA test results obtained from the 506 goats were different from those of the clinical examination, with 226 (44.7 %) seropositives against 221 (43.7 %) gestation with clinical diagnostic. The serological test results showed a few number of false positives 34 (6.7 %) and false negatives 39 (7.7 %). Indeed, nearly all the false negatives were female of less than 28 days of gestation. The entire false positive female had corpus luteum but 7 of them (18%) had also milk in the mammary gland. The sensitivity and the specificity of the test are respectively 84.6 and 86.3 %. The accuracy of the test is 87.8 %.
These results showed that the sensitivity and specificity of the test depends on the stage of gestation. PAG "sandwich" ELISA could then be suitable for pregnancy diagnosis in African dwarf goat as early as from day 28 after insemination.

KEYWORDS: Enzyme link immune assay, pregnancy associated glycoprotein, pregnancy, early diagnosis, African dwarf goat.

1 INTRODUCTION

During recent years, the imperatives of control of the reproduction in farms to increase the profitability of herds have led researchers and breeders to look more closely at different pregnancy diagnostic methods [1]. Indeed, pregnancy diagnosis is of great economic importance [2] and in the African pastoral economy, small ruminants (sheep and goats) have an important place because they intervened for 17% in population coverage of protein requirements [3]. Sub-Saharan Africa has 52.6 % of the global livestock of small ruminants that is 29.2 % of goats [4]. Their breeding is one of the main sources incomes of populations whose individual consumption of meat products is in constant growth [5].

Indeed, pregnancy diagnosis allows early detection of pregnancy and it monitoring, detection of unsuccessful mating and artificial insemination, identification of infertility cases and to do reforms in time [1]. There are several pregnancy diagnosis methods which different according to their earliness, sensitivity, specificity, ease of implementation and cost. In goats we

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have radiography, abdominal palpation, ultrasonography, hormonal assays and pregnancy specific or associated glycoprotein's assay [6].

Radiography is use to diagnose pregnancy with high accuracy from the 70^{th} day of gestation. However, this method cannot be widely used because of its high cost and especially the risk of environmental pollution [7]. Ultrasonography has high accuracy for pregnant females, but requires specialized personnel and the equipment has a relatively high cost [1], [7]. Hormones assays such as progesterone is a method of early diagnosis of pregnancy (day 21 in goats). However, the effectiveness of this method is subject to the knowledge of date of mating and the last heat of the female [1], [8].

Also, other known specific proteins such as protein B or Sasser Pregnancy Specific Protein B (PSPB), Pregnancy Serum Protein PSG-60 and Pregnancy Associated Glycoproteins (PAG) are used as pregnancy indicator or tool to investigate placental function in ongoing or endangered pregnancies [8], [9], [10], [11]. PAG´s was isolated, purified and characterized by Zoli and col [12] and permits early diagnosis of pregnancy in ruminants [13]. PAG molecules belong to the aspartic proteinase (AP) superfamily [14], their levels in peripheral circulation of pregnant females depends on the species, breed, sex of the kid, calving number [15] and litter size [16].

Although these methods are already available in developed countries for some specialized breeds whose production justifies the cost of the test, very little has been done for local goat breeds of Cameroon.

In this study, we describe the ability of develop Enzyme Link Immune Assay (ELISA) to detects pregnancy associated glycoprotein's and also to discriminate between pregnant and non-pregnant females of African dwarf goats. The test will be evaluated and compared to a post-mortem clinical pregnancy examination.

2 MATERIALS AND METHODS

2.1 TITRATION OF POLYCLONAL ANTI CAPAG IGG

After the extraction of PAG from fetal cotyledons according to the protocol of Garbayo and col [17] and production of anti caPAG antibodies [18], serial dilutions of anti caPAG IgG bind to biotin or not have permit the determination of their dilutions that will be used in the "sandwich" ELISA test.

2.2 EVALUATION OF THE ELISA TEST FOR EARLY PREGNANCY DIAGNOSIS

In other to make a screening after the development of the ELISA test for early diagnosis of pregnancy in African dwarf goat, the trial was carried out on 506 blood samples collected from goat bled in illegal small ruminant's slaughterhouse of the Dschang town. Blood collected was left at room temperature for a period of 12 to 24 hours, and the serum collected was stored in hemolysis tubes at -20°C until analysis. In case of pregnancy, the age of the fetus was determined by the following formula : $L = X^2 + 2X$ where L is the length of the fetus (in cm) comprise between the top of the occipital joint and the base of the tail, and X is the age of the fetus in months [19]. Clinical informations such as the state of the uterus (presence or absence of the corpus luteum), the state of breasts (presence or absence of milk) were also collected.

The sensitivity, the specificity and the accuracy of the test were determined by the following formula [1]:

$$\text{Sensitivity} = \frac{\text{Number of females true positive to test}}{\text{Total number of females effectively pregnant}} \times 100$$

$$\text{Specificity} = \frac{\text{Number of female's true negative to test}}{\text{Total number of females effectively non pregnant}} \times 100$$

$$\text{Accuracy} = \frac{\text{True positive + true negative to test}}{\text{Total number of females tested}} \times 100$$

2.3 STATISTICAL ANALYSIS

Statistical analysis was carried out by using the Chi Square test. The Statistical significance was considered at the $P < 0.05$ level. The software used was SPSS 20.

3 RESULTS

3.1 POST-MORTEM DIAGNOSIS OF PREGNANCY

Post mortem diagnosis showed that out of 506 goats slaughtered almost half of them (43.7 %) were pregnant (Table 1).

Table 1: Pregnant statue of goat post mortem

	Post mortem diagnostic		
	positive	**Negative**	**Total**
Number of females	221	285	506
Percentage (%)	43,7 [a]	56,3 [b]	100

Numbers affected by different letter are significantly different at the 5% significance level

3.2 POST-MORTEM DIAGNOSIS ACCORDING TO PREGNANCY STAGE

The distribution of pregnant female's diagnosed postmortem based on the pregnancy duration is presented in table 2. It shows that the slaughtered pregnant females are mostly in early gestational period (0 to1 months). They stand for 38.5% of the total pregnant female. This rate is followed by that of the females of 2 to 3 months pregnancy (23.1% 9) ($P < 0.05$).

Table 2: Post-mortem diagnosis of pregnancy according to pregnancy stage

Pregnant female	**Pregnancy stage (Month)**					
	0 to 1	**1 to 2**	**2 to 3**	**3 to 4**	**4 to 5**	**Total**
Effectif	85	44	51	33	8	221
percentage	38,5 [a]	19,9 [bc]	23,1 [b]	14,9 [c]	3,6 [d]	100

Numbers affected by the same letter are not significantly different at the 5% significance level

3.3 SEROLOGICAL DIAGNOSIS OF PREGNANCY ACCORDING TO POST MORTEM DIAGNOSIS

Out of 221 pregnant females diagnosed postmortem 187 (37 %) are positive to serological test. Indeed, there are 6.7% false negative to serological ELISA, ($P < 0.05$). In the other hand, out of 285 non-pregnant female's postmortem, 246 (48.6%) are negative to serological test, hence there are 7.7% false positives ($P < 0.05$) and the false positive rate is slightly above the false negatives.

Table 3: Serological diagnosis of pregnancy based on post mortem diagnosis

Post mortem diagnosis	**ELISA diagnosis**					
	Positive		**Negative**		**Total**	
	n	%	n	%	N	%
Positive	187	37,0 [a]	34	6,7 [b]	221	43,7
Negative	39	7,7 [a]	246	48,6 [b]	285	56,3
Total	226	44,7	280	55,3	506	100

n = number of female, % = percentage

Horizontal numbers affected by different letter are significantly different at the 5% significance level

3.4 SEROLOGICAL DIAGNOSIS BASED ON THE PREGNANCY STAGE

Serological ELISA test shows that there are pregnant females who are seronegative to pregnancy test and that these females have in majority less than 1 month pregnancy. Indeed, there are 33 false negatives (14.9%) between 0 and 1 month

of gestation (more precisely less tha 28 day) and 1 (0.5%) between 1 and 2 months (P <0.05). All females of 2 to 5 months of pregnancy are positive to the pregnancy test (Table 4).

Table 4: Serological diagnosis based on the pregnancy stage

ELISA diagnosis	Pregnancy length (Month)											
	0 to 1		1 to 2		2 to 3		3 to 4		4 to 5		Total	
	n	%	n	%	n	%	n	%	n	%	n	%
Positive	53	24	42	19	51	23,1	33	14,9	8	3,6	187	84,6
Negative	33	14,9[a]	1	0,5[b]	0	0	0	0	0	0	34	15,4
Total	86	38,5	43	19,9	51	23,1	33	14,9	8	3,6	221	100

n = number of female, % = percentage

Numbers affected by different letter are significantly different at the 5% significance level

3.5 Number Of False Positives According To The Presence Or Absence Of The Corpus Luteum And Milk In The Mammary Glands

more than 3/4 of false positives (82%) had a corpus luteum and milk in the mammary glands, while less than one quarter (18%) had no lutea that can afford to assume an early gestation although serological diagnosis is positive (P <0.05). However, all the false positives had milk in the mammary gland (Table 5).

Table 5: Distribution of false positives based on the presence or absence of the corpus luteum and milk in the mammary glands

Milk in mammary gland	Falses positives					
	Corpus luteum					
	Present		Absent		Total	
	n	%	n	%	N	%
Present	32	82,0[a]	7	18,0[b]	39	100
Absent	0	0	0	0	0	0
Total	32	82,0	7	18,0	39	100

Numbers affected by the same letter are not significantly different at the 5% significance level

3.6 Number Of False Negatives According To The Pregnancy Stage

Almost all the false negatives were recorded at an early gestational age (0-1 month) (97 %), with very little pregnancy of more than a month (3 %). The majority of false negatives is therefore between 0 and 1 month of pregnancy (Table 6) and precisely below 28 days (P <0.05).

Table 6: Number of false negatives according to the stage of pregnancy

Falses negatives	Pregnancy stage (Month)		
	0 to 1	1 to 2	Total
Effectif	33[a]	1[b]	34
percentage	97.0	3.0	100

Numbers affected by different letter are significantly different at the 5% significance level

3.7 Serological Test Sensitivity Depending On Pregnancy Stage

ELISA serological test sensitivity varies with duration of gestation (Table 7). In fact, the sensitivity was lower for pregnancy of less than one month (61.6%). For pregnancy of more than a month, the sensitivity is greater than or equal to 97.7%.

Table 7: Serological test sensitivity depending on pregnancy stage

Quality of the serological test	Pregnancy stage (Month)		
	0 to 1	1 to 2	>2
Sensitivity (%)	61.6	97.7	100

3.8 SENSITIVITY, SPECIFICITY, AND ACCURACY OF THE SEROLOGICAL TEST

Goat PAG is detectable in the serum of African dwarf goats with a sensitivity of 84.6% and a specificity of 86.3%. The test is 85.6% reliable (total accuracy) for the diagnosis of pregnancy in goat (Table 8).

Table 8: Sensitivity, specificity, and accuracy of the serological test

Sensitivity	Specificity	accuracy +	accuracy -	Total accuracy
84,6%	86,3%	82,7%	87,8%	85,6%

4 DISCUSSION

The semi-purified protein obtained was used for the production a specific antiserum which involved the development of an enzyme linked immunosorbent assay (sandwich ELISA) for the detection of caPAG in the maternal circulation in view of early diagnosis of pregnancy in goat. The evaluation of this test was done on 506 sera collected from African dwarf goats bled illegally in small ruminants slaughter house of the city of Dschang / Cameroon. Thus, the clinical diagnosis based on a clear observation of the fetus was used to determine the number of pregnant females and the duration of pregnancy. The results were then compared with that of the serological test.

Pregnancy diagnosis by serological test (ELISA- sandwich) gave significantly different results from those of the post-mortem diagnosis, with less false positives (34) than false negatives (39) out of 506 goats tested. Indeed serological test sensitivity was 84.6% and specificity was 86.3%. This sensitivity is lower than that obtained by Gonzalez and col, [20], Sousa and col [17], Beckers [21], with RIA method. The positive accuracy of the diagnoses was 82.7% and the negative accuracy was 87.8%. These values are lower than those found by Zoli and col [13] in cattle which were 91.77% and 97.90% respectively by the RIA method.

However the test conducted by these authors was a RIA test and the test was compared to a clinical test which was recto-abdominal palpation on the days 40 of pregnancy, on well followed animals. While the test we have conducted was compared to a post-mortem clinical diagnosis on slaughter goats where gestations of less than 1 month were many, giving rise to a high rate of false negative (6.7 %) to the serological test. These false negatives therefore pose the problem of the test ability to detect early pregnancies. That is, pregnancy of less than a month. Indeed all the 34 pregnant goats declared negative had a gestation of less than 28 days. One might think that below this stage of gestation the concentration PAG produced is still low and this resulted in a lower sensitivity for gestation of less than a month. Whereas beyond this range, the sensitivity of the test was 97.7 to 100% as that obtained by Gonzalez and col [20] at day 26 of pregnancy on Alpine goats and El Battawy and col [22], on buffalo cow at day 28 after insemination.

Among the false negative, 82% had a corpus luteum and also milk in the mammary glands. The presence of the corpus luteum can indicate physiological conditions of a normal cycle (luteal phase) [23]. However, with the presence of milk in the mammary glands and the seropositive status of these females, one might think that these females were victims of embryonic mortality, early abortion or premature death of the kid. Breukelman and col [8] mentioned that after pregnant loss, PAG can still be detected in maternal circulation for many days. Also after birth, PAG´s are still detected in maternal circulation 30 days after in small ruminant [24], [25], and more than 60 days in cattle [26] [27] [28] . At the other hand, PAG concentrations during the early fetal period are also related to milk production [29]. Furthermore, 18% of false positives had no luteum but had milk in the mammary glands. These exclude any hypothesis of early pregnancy, but do not rule out a persistence of caPAG due to a previous pregnancy or a high basal level due to individual variations [13], [30], [31].

5 CONCLUSION

Detection of pregnancy associated glycoprotein by ELISA sandwich is a sensitive and specific method of diagnostic of gestation in African dwarf goat. Thus the sensitivity is influences by the stage of gestation and it is lower when gestation is

less than 28 days. In the other hand, the test offers the advantages that it requires a single plasma sample for early pregnancy diagnosis and could then be suitable for pregnancy diagnosis in Africa dwarf goat as early as day 28 after insemination.

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