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PREGNANCY DIAGNOSIS IN SHEEP

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OF

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List of abbreviations

bpm ....beat per minute

BSA..bovine serum albumin

boPAG..................................bovine pregnancy-associated glycoprotein

caPAG...................................caprine pregnancy-associated glycoprotein

cpm..counts per minute

eCG ....equine chorionic gonadotrophin

^{125}I ....ioden 125

IgG ....immunoglobulin G

i.m. ....intramuscular(ly)

IU ....international unit

KDa.......................................kilo dalton

M ....molar

mg ....milligram

MHz ....mega hertz

ng/mL .... nanogram per milliliter

ovPAG...................................ovine pregnancy-associated glycoprotein

P ....probability

P4 ....progesterone

PAG-RIA ....pregnancy associated glycoprotein-radioimmunoasssay

PSPB ....pregnancy-specific protein B

PEG.......................................polyethelen glycol

RIA..radioimmunoassay

SD ....standard deviation

INTRODUCTION *(Karen et al., 2001)*

Accurate diagnosis of early pregnancy is a key factor for successful reproductive management in sheep farms. Among the numerous methods used for pregnancy diagnosis in sheep, a few methods can diagnosis early pregnancy. However, these methods are either impractical or not specific for pregnancy. Recently, pregnancy-associated glycoproteins (PAG) have been isolated from domestic ruminant placentas and radioimmunoassays have been developed for detecting PAG in the maternal blood or in the milk of pregnant animals. These radioimmunoassays have been used accurately for detecting early pregnancy in cattle and goats. However, there is no data about the accuracy of pregnancy associated glycoprotein-radioimmunoassay (PAG-RIA) for early pregnancy diagnosis in sheep. Transrectal ultrasonography has been recommended as a practical test for early pregnancy diagnosis in sheep. The variable accuracy of transrectal ultrasongraphy for early pregnancy diagnosis in sheep led us to study the factors, which could improve the accuracy of the technique and to investigate the false ultrasonographic pregnancy diagnoses.

THE AIM OF THE THESIS

This work was undertaken to find the most accurate method for early pregnancy diagnosis in Awassi x Merino ewes.

Three studies were carried out to achieve the purpose of the thesis

1. Early pregnancy diagnosis in sheep by progesterone and pregnancy-associated glycoprotein tests *(Karen et al. 2003 a)*.

This is the first study to evaluate the accuracy of the pregnancy-associated glycoprotein radioimmunoassay (PAG-RIA) test for detecting early pregnancy in sheep and to compare it with that of progesterone radioimmunoassay (P4-RIA) test.

1.1. Materials and Methods

One hundred and eighty-two Awassi x Merino ewes were synchronized for estrus by insertion of intravaginal sponges containing 30-mg flurogestone acetate for 14 days at the

beginning of the breeding season. At the time of sponge removal, all ewes were administered 600 IU eCG i.m. All ewes were inseminated twice with fresh semen at 48 and 56 h after sponge removal. Day of insemination was considered as Day 0 for calculating the gestational age. Blood sample was collected from each ewe at Days 0, 18, 22, 29, 36 and 50 after AI.

Plasma concentrations of P4 at Days 0 and 18 after AI were measured by a double-antibody radioimmunoassay. Ovine PAG concentrations at Days 22, 29, 36 and 50 after AI were determined by a heterologous double-antibody radioimmunoassay using boPAG 67 KDa as tracer and standard and rabbit antiserum raised against a mixture of caPAG 55+59 KDa as the first antibody. The discriminatory value for diagnosis of pregnancy by P4 and PAG-RIA tests was $\geq$ 1ng /mL.

Based on the lambing data and any observed sign like abortion, the accuracy for detecting pregnant (sensitivity) and non-pregnant ewes (specificity), and predictivity of both tests were calculated. The exact binomial test was used to compare the sensitivity and specificity of the P4 test at Day 18 with the PAG test at Days 22, 29, 36 and 50 after AI.

1.2. Results

The accuracies of P4 and PAG-RIA tests for pregnancy diagnosis are shown in Table 1. The sensitivity of P4 test at Day 18 after AI was 100 %, while the specificity of the test was 95.6 %, because 7 non-pregnant ewes had P4 levels higher than 1 ng/mL (false positive diagnoses). Two of these ewes had high P4 level (>1 ng/mL) at Day 0. Regarding PAG-RIA test, the sensitivity at Day 22 was 93.5 %, because 2 pregnant ewes had ovPAG level lower than the threshold for diagnosis of pregnancy. At Days 29, 36 and 50 after AI, the sensitivity of the test reached 100 %. The specificity of the test was 100% at Days 22, 36 and 50, while it was 99.2% at Day 29, because one non-pregnant ewe had PAG level (1.02 ng/mL) slightly higher than the threshold for diagnosis of pregnancy. The specificity of PAG-RIA test at Days 22, 36 and 50 after AI was significantly ($P< 0.05$) higher than that of P4-RIA test. On the other hand there was no significant difference in the sensitivity of both tests for detecting pregnant ewes.

2. Accuracy of transrectal ultrasonography for determination of pregnancy in sheep: effect of fasting and handling of the animals *(Karen et al. 2003 b)*

This field study was designed to investigate the effect of fasting ewes before scanning and lifting up their abdominal wall during scanning on the accuracy of transrectal ultrasonography for pregnancy diagnosis.

2.1. Material and Methods

Four flocks (A, B, C and D) of Awassi x Merino ewes (n=1247), aged 0.7 to 10 years, were synchronized for estrus and were artificially inseminated. Two weeks later, fertile rams were kept with ewes of flocks A, B and C (n=949) to mate those returned to estrus, while ewes of flock D (n=298) were re-inseminated 17 days after the first insemination.

All ewes were scanned by B-mode real-time ultrasound scanner (Aloka SSD-500) equipped with a 5 MHz linear-array transducer. The probe of the scanner was taped to a plastic rod to control the manipulation of the transducer inside the rectum. The same operator who had trained for ultrasonographic pregnancy diagnosis in small ruminants carried out all the transrectal ultrasonograhic examinations. A total of 1713 ultrasound scans were made in 1247 ewes, whereas ewes of flock A, B and C were scanned on four separate occasions after separating the diagnosed pregnant ones, while ewes of flock D were scanned once. The results of ultrasound scanning (*n*=949) carried out on the first occasion on ewes of flocks A, B and C were attributed to Group 1. Ewes belonging to this group were scanned in a standing position in the milking parlor. The scans (*n*=764) made during the second, third and fourth occasions on ewes of flocks A, B and C and all ewes of flock D was attributed to Group 2. Ewes belonging to this group were scanned by using the same technique applied in the animals of Group 1. However, the ewes were fasted for 12 hours prior to scanning and their abdominal wall in front of udder was lifted up by the assistant's hands during scanning.

Depending on the stage of pregnancy, the recognition of allantoic fluid, embryo proper, placentome or fetus was considered as a positive sign of pregnancy.

The gestational age at the time of scanning was calculated either by considering day of insemination as day 0 (for ewes conceived after AI) or by subtracting the interval from scanning till lambing from 150 day (for ewes conceived by natural mating).

To study the influence of the age of the ewes on the accuracy of transrectal ultrasonography, each of the two groups was subdivided into two subgroups; the first included young ewes (aging (0.7-2 years) and the second included ewes aged between more than 2 and 10 years.

Based on the lambing performance of tested ewes, the sensitivity, the specificity, and the positive and negative predictive values of the transrectal ultrasonography in both groups and their subgroups were calculated. The sensitivity and the specificity of transrectal ultrasonography obtained in Groups 1 and 2 and in their subgroups were compared by Fisher`s exact (total number of the ewes < 200) test and Pearson`s Chi-square test with Yate`s continuity correction (total number of the ewes >200).

2.2. Results

The accuracies of transrectal ultrasonography for pregnancy diagnosis in Groups 1 and 2 are shown in Table 2. The sensitivity of transrectal ultrasonography for detecting pregnant ewes in Group 2 was significantly higher than that of the test in Group 1 in all periods of scanning (Table 2). No significant differences in the specificity of the test were observed between the two groups with the exception of scan periods Days 41 to 50, during which specificity of the test in Group 1 was significantly ($P< 0.01$) higher than that of the test in Group 2 (Table 2).

In Group 1, the sensitivity of the test at Days 31 to 41 and 41 to 50 was significantly ($P<0.0001$, $P= 0.002$, respectively) higher than that of the test at Days 18 to 24 of gestation. Furthermore, a significant ($P = 0.003$) higher sensitivity was made by the test at Days 31 to 40 than that at Days 25 to 30 of gestation. In Group 2, The sensitivity of the test at Days 25 to 30, 31 to 40 and 41 to 50 was significantly ($P< 0.0001$) higher than that of the test at Days 18 to 24 of gestation (Table 2).

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Regarding the effect of the age of the ewes, the sensitivity of the test was significantly higher in young ewes of Group 1 at scan periods Days 18 to 24 and Days 41 to 50 of gestation (Table 3). In addition, the sensitivity of the test increased in young ewes when scanning took place at a more advanced stage of pregnancy, reaching the maximum at scan period Days 41 to 50. By contrast, in older ewes the sensitivity of the test decreased after Day 40 of gestation (Table 3). The accuracies of the test in the two age subgroups of group 2 were not significantly different (Table 4).

3. Evaluation of false transrectal ultrasonographic pregnancy diagnoses in sheep by measuring plasma level of pregnancy-associated glycoproteins *(Karen et al. 2003 c)*

The aim of the present study was to investigate to what extent pregnancy diagnoses made by transrectal ultrasonography could be confirmed by the measurement of plasma ovPAG concentrations.

3.1. Material and Methods

A total of 424 awassi x merino ewes were synchronized for estrus. All ewes were artificially inseminated and the day of insemination was considered as day 0 for calculating the gestational age.

Transrectal ultrasonographic examinations were conducted in two experiments by using a real-time B-mode ultrasound scanner (Aloka SSD-500) equipped with a 5 MHz linear-array transducer. In Experiment 1, 156 ewes were scanned at Days 29, 36 and 50 after AI in a standing position in the milking parlor. Recognition of allantoic fluid at Days 29 and 36 of gestation was considered as a positive sign of pregnancy. At Day 50, the recognition of the fetus or placentomes was used as a criterion for a positive pregnancy diagnosis. In Experiment 2, 268 ewes were scanned by the same operator using the same technique applied in Experiment 1, but ewes were fasted for 12 hours prior to scanning and their abdominal walls was lifted up by assistant's hands during the scanning.

After each transrectal ultrasonography, blood sample was withdrawn from each ewe in both experiments. Concentrations of plasma ovPAG were measured by the heterologous

double-antibody radioimmunoassay. The discriminatory value for diagnosis of pregnancy by PAG-RIA test was ≥ 1ng /ml.

Based on the lambing performance or any observed sign like abortion, the results of transrectal ultrasonography and PAG-RIA test were arranged into: correct positive diagnoses (pregnant), incorrect positive diagnoses (false positive diagnosis), correct negative diagnosis (non-pregnant) and incorrect negative diagnosis (false negative diagnosis). From these values, the sensitivity, the specificity and the predictivity of transrectal ultrasonography and PAG-RIA test were calculated. The exact binomial test was used to compare the sensitivity and the specificity of transrectal ultrasonography with those of PAG-RIA test.

3.2. Results

In Experiment 1, all lambed (n=28) or aborted (n=3) ewes were diagnosed as pregnant by PAG-RIA at Days 29, 36 and 50. However, by transrectal ultrasonography pregnancy was detected only in 16 ewes (51.6%) at Day 29, 13 ewes (41.9 %) at Day 36 and in 15 ewes (51.7 %) at Day 50 of gestation (Table 5). The level of sensitivity of PAG-RIA test for detecting pregnant ewes was significantly (P<0.001) higher than that of transrectal ultrasonography during each of examination days. Forty-seven false negative diagnoses (15 at Day 29, 18 at Day 36 and 14 at Day 50) were made in 24 ewes by transrectal ultrasonography.

With one exception, all non-lambing ewes had ovPAG concentration lower than the threshold of pregnancy at Days 29, 36 and 50 after AI. This ewe had ovPAG concentration slightly higher (1.02 ng/mL) than the threshold of pregnancy at Day 29 and was ultrasonographically diagnosed non-pregnant during each of the scanning days. By transrectal ultrasonography, non-pregnancy was diagnosed in 104 ewes (83.2 %) at Day 29, 110 ewes (88.7 %) at Day 36 and in 116 ewes (97.4%) at Day 50 (Table 5). The level of specificity of the PAG-RIA test was significantly (P<0.001) higher than that of transrectal ultrasonography at Days 29 and 36 of gestation. The level specificity of transrectal ultrasonography at Day 50 was significantly higher than that of the test at Days 29 (P= 0.0003) and 36 (P= 0.0127) of gestation (Table 5).

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In Experiment 2, all lambing (*n*=29) or aborted (n=*3*) ewes had ovPAG level higher than the threshold for diagnosis of pregnancy during the examined periods. However, by transrectal ultrasonography pregnancy was diagnosed in 26 ewes (81 %) at Day 24, 31 ewes at Days 29 and 34 (96.8 %) of gestation (Table 6). The level of sensitivity of PAG-RIA test was significantly ($P<0.05$) higher than that of transrectal ultrasonography only at Day 24.

Eight false negative diagnoses (6 at Day 24, 1 at Day 29 and 1 at Day 34, respectively) were made by transrectal ultrasonography in 7 ewes during the examined period.

With two exceptions, all non-lambing ewes had ovPAG concentrations lower than the threshold level established for diagnosis of pregnancy. The two ewes were diagnosed as non-pregnant by transrectal ultrasonography during each of the scanning days. Thirteen false positive diagnoses (7 at Day 24, 3 at Day 29 and 3 at Day 34, respectively) were made by transrectal ultrasonography in 7 ewes during the study period.

In both experiments, all ewes with false negative transrectal ultrasonographic diagnoses had ovPAG levels higher than the threshold for diagnosis of pregnancy ($\geq$ 1ng /ml). Therefore these ewes were pregnant at the time of transrectal ultrasonography. In addition, all ewes with false positive transrectal ultrasonograhic diagnoses had ovPAG level lower than the threshold for diagnosis of pregnancy, therefore theses ewes were not pregnant at the time of transrectal ultrasonography.

CONCLUSIONS

Results of the present thesis show that radioimmunoassay of the plasma progesterone at Day 18 after AI detects all pregnant ewes. However the specificity of the test for detecting non-pregnant ewes is relatively low, because plasma level of progesterone $\geq$ 1 ng /mL indicates a functional corpus luteum which associates with pregnancy, pathological cases such as pyometra and hydrometra, luteal cysts, embryonic mortality or irregular estrus cycles. The heterologous double-antibody radioimmunoassay using boPAG as standard and tracer and the antiserum raised against caPAG 55+59 as the first antibody accurately determines pregnant and non-pregnant ewes from Day 22 after AI.

Because PAG are secreted from placenta therefore, its presence in maternal plasma in a level higher than the threshold indicates the presence of pregnancy. Therefore, PAG-RIA test is more specific than the progesterone test for detecting non-pregnant ewes and unlike progesterone test, it can differentiate between pregnancy and prolonged inter-estrus interval. Our results indicate that PAG-RIA test is more accurate than transrectal ultrasonography (5 MHz) for detecting pregnant ewes at Day 24 of after AI. When the day of insemination is known, a single blood sample is required for PAG-RIA test to accurately differentiate between pregnant and non-pregnant ewes from Day 22 after AI. The potential using the PAG-RIA test in a modified kit form will enable the sheep producer to apply the test in the farm and over come the time delay and the hazard of radiation.

From the results of the thesis, we can also conclude that 5 MHz transrectal ultrasonography is an accurate and practical method for pregnancy diagnosis in Awassi x Merino ewes from Day 29 after AI and when ewes are fasted for 12 hours before scanning and their abdominal wall is lifted up during scanning. In addition, using the embryo proper, fetus or placentome with allantoic fluid as positive signs of pregnancy is recommended to avoid the false positive diagnoses made by transrectal ultrasonography.

New findings of the thesis

- The heterologous radioimmunoassay for pregnancy associated glycoproteins (PAG) is a reliable and accurate method for determining pregnant and no-pregnant ewes from Day 22 after breeding.

- Fasting Awassi x Merino ewes for 12 hours prior to scanning and lifting up the abdominal wall of the animal during scanning greatly improve the accuracy of transrectal ultrasonography for diagnosing pregnancy.

- Transrectal ultrasonography with a 5 MHz transducer is an accurate technique for pregnancy diagnosis in Awassi x Merino ewes from Day 29 of gestation.

- PAG-RIA test is more accurate than transrectal ultrasonography for diagnosing early pregnancy in Awassi x Merino ewes. In addition the test is more specific than progesterone test for detecting non-pregnant ewes.

Table 1: Sensitivity (Se), specificity (Sp) and predictive values (+PV, -PV) of the progesterone (P4) and pregnancy-associated glycoprotein (PAG) tests in Awassi x Merino ewes

Days of pregnancy	No. of ewes (n)	Pregnancy test	a	b	c	d	Se (%)	Sp (%)	+PV (%)	-PV (%)
18	182	P4	31	7	144	0	100	95.4^a	81.5	100
22	182	PAG	29	0	151	2	93.5	100^b	100	98.7
29	156	PAG	31	1	124	0	100	99.2	96.8	100
36	155	PAG	31	0	124	0	100	100^b	100	100
50	148	PAG	29	0	119	0	100	100^b	100	100

a: correct positive diagnosis (pregnant)
b: incorrect positive diagnosis (non-pregnant)
c: correct negative diagnosis (non-pregnant)
d: incorrect negative diagnosis (pregnant)
^{ab}P< 0.05

Table 2. Sensitivity (Se), specificity (Sp), and predictive values (+PV, -PV) of transrectal ultrasonography for pregnancy diagnosis in groups 1 and 2 of Awassi x Merino ewes

Grouping and evaluation	Group 1 (n=949)				Group 2 (n=764)			
	Days of gestation				Days of gestation			
	18-24 (n=161)	25-30 (n=84)	31-40 (n=352)	41-50 (n=352)	18-24 (n=113)	25-30 (n=228)	31-40 (n=248)	41-50 (n=175)
a	14	11	69	28	23	99	109	123
b	7	4	20	16	5	7	7	8
c	90	46	223	280	58	114	123	40
d	50	23	40	28	27	8	9	4
Se (%)	$21.8^{\text{e IK}}$	$32.3^{\text{g J}}$	$63.3^{\text{gI J}}$	$50.0^{\text{g K}}$	$46.0^{\text{f L}}$	$92.5^{\text{h M}}$	$92.3^{\text{h M}}$	$96.8^{\text{h M}}$
Sp (%)	92.7	92.0	91.7	94.6^{e}	92.1	94.2	94.6	83.3^{f}
+PV (%)	66.6	73.3	77.5	63.6	82.1	93.3	93.9	93.8
-PV (%)	64.2	66.6	84.7	90.9	68.2	93.4	93.1	90.9

a: correct positive diagnosis (pregnant), b: incorrect positive diagnosis (non pregnant), c: correct negative diagnosis (non-pregnant), d: incorrect negative diagnosis (pregnant).
$^{e, f}$ (P<0.01) , $^{g, h}$ (P< 0.0001): significant difference between the two groups.
Percentages with the same capital letter superscript in group 1 are significantly different (P<0.0001, P= 0.003, P=0.002 for I, J and K respectively).
$^{L, M}$ (P< 0.0001): significant difference within group 2.
Figures in parentheses indicate the numbers of ultrasonographic examinations.

Table 3. Effect of age of ewes on the accuracy of transrectal ultrasonography for pregnancy diagnosis (group 1)

Days of gestation	Age of ewes (year)	No. of ewes	a	b	c	d	Se %	Sp %	+PV %	-PV %
18-24	0.7 to 2	54	9	4	35	6	60.0 [e]	89.7	69.2	85.3
	>2 to 10	107	5	3	55	44	10.2 [f]	94.8	62.5	55.5
25-30	0.7 to 2	46	4	2	38	2	66.6	95.0	66.6	95.0
	>2 to 10	38	7	2	8	21	25.0	80.0	77.7	27.5
31-40	0.7 to 2	83	17	3	56	7	70.8	94.9	85.0	88.8
	>2 to 10	269	52	17	167	33	61.1	90.7	75.3	83.5
41-50	0.7 to 2	60	7	2	51	0	100 [g]	96.2	77.7	100
	>2 to 10	292	21	14	229	28	42.8 [h]	94.2	60.0	89.1

a: correct positive diagnosis (pregnant), b: incorrect positive diagnosis (non pregnant), c: correct negative diagnosis (non-pregnant), d: incorrect negative diagnosis (pregnant). Se: sensitivity, Sp: specificity, +PV: Positive predictive value, -PV: negative predictive value.

[e, f] $P<0.001$.
[g, h] $P <0.05$.

Table 4. Effect of age of ewes on the accuracy of transrectal ultrasonography for pregnancy diagnosis (group 2)

Days of gestation	Age of ewes (year)	No. of ewes	a	b	c	d	Se %	Sp %	+PV %	-PV %
18-24	0.7 to 2	14	3	1	3	7	30.0	75	75.0	30.0
	>2 to 10	99	20	4	55	20	50.0	93.2	83.3	73.3
25-30	0.7 to 2	49	26	3	19	1	96.2	86.3	89.6	95.0
	>2 to 10	179	73	4	95	7	91.2	95.9	94.8	93.1
31-40	0.7 to 2	32	19	1	11	1	95.0	91.6	95.0	91.6
	>2 to 10	216	90	6	112	8	91.8	94.9	93.7	93.3
41-50	0.7 to 2	38	31	2	4	1	96.8	66.6	93.9	80.0
	>2 to 10	137	92	6	36	3	96.8	85.7	93.8	92.3

a: correct positive diagnosis (pregnant), b: incorrect positive diagnosis (non pregnant), c: correct negative diagnosis (non-pregnant), d: incorrect negative diagnosis (pregnant). Se: sensitivity, Sp: specificity, +PV: Positive predictive value, -PV: negative predictive value.

Table 5. Sensitivity, specificity and predictive values of transrectal ultrasonography for early pregnancy diagnosis in Awassi x Merino ewes (Experiment 1).

Days of pregnancy	No. of ewes	a	b	c	d	Se %	Sp %	+ PV %	-PV %
29	156	16	21	104	15	51.6	83.2 [e]	43.2	87.4
36	155	13	14	110	18	41.9	88.7 [f]	48.1	85.9
50	148	15	3	116	14	51.7	97.4 [e f]	83.3	89.2

a: correct positive diagnosis (pregnant), b: incorrect positive diagnosis (non-pregnant), c: correct negative diagnosis (non-pregnant), d: incorrect negative diagnosis (pregnant), Se: sensitivity, Sp: specificity, +PV: positive predictive value, -PV: negative predictive value.
Percentages within a column and with the same superscript are different ([e] $P=0.0003$; [f] $P= 0.0127$, respectively).

Table 6. Sensitivity, specificity, and predictive values of transrectal ultrasonography for early pregnancy diagnosis in Awassi x Merino ewes (Experiment 2).

Days of pregnancy	No. of ewe	a	b	c	d	Se %	Sp %	+ PV %	-PV %
24	268	26	7	229	6	81	97	78.7	97.4
29	268	31	3	233	1	96.8	98.7	91.1	99.5
34	251	31	3	216	1	96.8	98.5	91.1	99.5

a: correct positive diagnosis (pregnant), b: incorrect positive diagnosis (non-pregnant), c: correct negative diagnosis (non-pregnant), d: incorrect negative diagnosis (pregnant), Se: sensitivity, Sp: specificity, +PV: positive predictive value, -PV: negative predictive value.

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