

ASSESSMENT OF PREGNANCY IN ESTRUS SYNCHRONIZED IRAQI EWES BY APPLICATION DIFFERENT DIAGNOSTIC METHODS AND A COMPARISON BETWEEN THEM

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Abstract

The aim of current study was to evaluate and compare sensitivity, specificity and accuracy of pregnancy associated glycoprotein (PAG- ELISA), progesterone (P4-ELISA) and ultrasonography testes for distinguish between pregnant and non pregnant Iraqi ewes at day 30 and 60 of pregnancy.

Eighteen synchronized Iraqi cross breed ewes were scanned by transrectal ultrasonography (6.5 MHz liner probe) at day 30 of pregnancy and transabdominal ultrasonography (3.5 MHz sector probe) at day 60 of pregnancy. After each scan blood were collected from jugular vein to confirm the values of ovine pregnancy associated glycoprotein (ovPAG) and progesterone (P4) by ELISA. The distinguish value for detecting pregnancy by sheep PAG ELISA kit, sheep P4 ELISA kit and ultrasonography, based on parturition data. The predictive value of positive and negative test, accuracy, sensitivity and specificity of the PAG- ELISA were 100%, 100%, 100%, 100% and 100% on both 30 and 60 of pregnancy respectively and the predictive value of positive and negative test, accuracy, sensitivity and specificity of the P4- ELISA were 100%, 100%, 100%, 100% and 100% on both 30 and 60 of pregnancy respectively, while the predictive value of positive and negative test, accuracy, sensitivity and specificity of ultrasonography were 100%, 62.5%, 83.3%, 76.9% and 100% at day 30 and 100%, 100%, 100%, 100% and 100% at day 60 of pregnancy respectively. It can be concluded that ovine pregnancy is reliably diagnosed at day 30 after mating by using sheep PAG- ELISA and sheep P4- ELISA, but PAG- ELISA consider the most reliable because to P4 from CL of pregnancy or from CL of other physiological or pathological sources.

Keywords: Iraqi sheep; ELISA; Ultrasonography; Progesterone; pregnancy associated glycoprotein; pregnancy diagnosis.

INTRODUCTION

Iraqi ewes can be considered seasonal polyestrous sheep and the activity of their reproductive aligns perfectly the ideal timeframe of a year where available food resources during spring (Hussain et al., 2017, Hatif and Younis, 2018a). Also Younis et al. (2019) observed estrus activity in some ewes in April. Generally sheep considered seasonal polyestrous animals and short day breeders (Younis and Hatif 2017 and Ajafar et al., 2022). The breeding season started in autumn and continued until mid or late winter, while anestrus period happening through spring and summer (Hatif and Younis 2018a and b). The beginning and period of breeding season may be effected via interaction between genetic factors and daylight (Al-Mutar, 2017 and Younis et al., 2020).

Estrus synchronization in ewes may be achieved via regulating the activity secretion of corpus luteum and ovulation (Aqwaan, 2023). Synchronization of estrus in

ewes can be achieved by administration the exogenous hormones such as progesterone (Tamer and Al-Hamedawi, 2013 and Al-Zubaidi, 2017) also by adding equine chorionic gonadotropin (Hussain, 2007, Al-Zubaidi, 2017 and Kadhimi and Hussain, 2014). The administration of equine chorionic gonadotropin with progesterone during both breeding and non breeding season can improve estrus response and gestation rate (Husseini et al., 2007). Synchronization of estrus can be achieved via different methods including daylight manipulation, ram effect and exogenous hormones administration (progesterone, eCG, prostaglandins, melatonin, kisspeptin and Bromocriptine) during both breeding and non breeding season in sheep (Tamer and Al-Hamedawi, 2013, Al-Hamedawi, et al., 2016, Al-Hamedawi, et al., 2020, Abas et al., 2022 and Kadhimi and Hussain, 2024a) and in goat (Kadhimi et al., 2014). The ram effect may be improve the effectiveness of progesterone and serve as substitution for eCG in sheep (Al-Mutar, 2017 and Hameed et al., 2021). During both

breeding and non breeding season the administration of progesterone has demonstrated to effect on estrus induction and synchronization in ewes (Abdul Hussain et al., 2017). Estrus response and lambing rate were higher significant in ewes using CIDR+Kisspeptin injection than those used CIDR alone (Abdul Kareem et al., 2021), but other authors show no significant difference between two groups when used same protocols (Kadhim and Hussain, 2024a).

Pregnancy maintain are clear economics factors in dairy animals since the majority of the pregnancy losses are observed during the first stages of pregnancy (García-Ispuerto et al., 2013; Commun et al., 2016). Therefore, early pregnancy diagnoses can be a very important tool that can be used in herd management to ensure good reproductive health of livestock herds (Green et al., 2005, Kaya et al., 2016). Diagnosis of pregnancy in animals uses four techniques that are Ultrasound, estrone sulfate, Milk /progesterone assay, and blood tests. An earlier pregnancy detection can be investigation by using trans rectal ultrasonography, but require expert operator and long time, it highlights that the Range of gestational detection by this process can be started between 30 to 50 days (Gonzalez- Bulnes et al ., 2010). the primate luteinizing hormone (P4) screening is the most accurate and sensitive as early as Days 17 to 19 (Karen et al., 2003). Nevertheless pregnancy hardly obtain an accurate diagnosis as a result of one or the other causes (Commun et al., 2016). Thus, a simple and reliable pregnancy diagnosis for animals particularly for domestic use has long been searched. Other method is used to diagnose pregnancy with the help of specific pregnancy-associated glycoproteins (PAGs). PAGs are considered key indicators for the purpose of early pregnancy diagnosis given that they are produced within the first few days of the pregnancy (Commun et al., 2016). The results of ELISA-PAG shown that it was an efficient, rapid, and suitable method for early pregnancy diagnosis in the sheep and it constituted a tool to reproductive biotechnologies ranging used from the 30th day of gestation in ewes. Thus, ELISA-PAG test may be considered as an alternative for rapid and accurate detection of PAG, as well as the identification non-pregnant ewes and it showed similar or better sensitivity as trans-rectal ultrasonography for pregnancy detection (de Miranda et al. 2017). There have been numerous studies in the field of reproduction science that have sought to analyse pregnancy-associated glycoprotein. These studies have not only information regarding early pregnancy diagnosis, but also foetal number, pregnancy viability, fetal sex (Karen et al., 2014; Karen et al., 2015; Piechotta et al., 2011; and Rovani et al., 2016).

Methods of pregnancy diagnosis

Ultrasonic techniques

transabdominal ultrasonography with a 3.5 MHz probe may accurately diagnose pregnancy in Balkhi ewes up to day 42 of gestation. When compared to necropsy exams or lambing, transabdominal ultrasonography had 100% specificity and accuracy in a different research

involving sheep at 39 days (Jones et al., 2016). In order to identify and track a pregnant sheep, real-time ultrasonography is a crucial tool. Jones et al. (2016), Stankiewicz et al. (2020), and Santos et al. (2021) are some of the studies that consider the uterine characteristic pictures, the existence of the embryo and embryonic vesicle, the embryo's heartbeat, and the presence of placentomes. For herd management purposes, early pregnancy identification is crucial, since 30–50% of embryos do not survive this first time (Chundekkad et al., 2020). Because pregnant sheep are a useful biological model, early pregnancy identification also allows for the rapid initiation of studies examining intrauterine development (Morrison et al., 2018). According to Bairagi et al. (2016), developing functional uteroplacental and fetoplacental circulation is one of the first processes that decides the pregnancy's outcome, placental angiogenesis starts on day 18 in sheep. In order to meet the needs of the developing foetus and ensure proper material exchange between the mother and the foetus, the placenta continues to develop throughout pregnancy. This development is closely related to the development of blood vessels in the placenta (Stankiewicz et al., 2020; Blaszczyk et al., 2020). Elmetwally and Bollwein (2017), Stankiewicz et al. (2020), and Santos et al. (2021) are among the few studies that have primarily examined sheep for blood flow in the umbilical, uterine, and foetal arteries. The umbilical chord is also included in the evaluation of foetal development (Troisi et al. 2018). Doppler ultrasonography can be detected in sheep umbilical artery hemodynamic measurements in non-invasive manner, and pharmacological-free (Tomasz et al., 2019). Brzozowska et al. (2022) found that trans rectal ultrasonography is a practical and convenient way to diagnose pregnancy in sheep. They also found that B-mode ultrasonography can be a simple way to confirm effective.

Hormones assay

Estrone sulphate, progesterone, and pregnancy-associated glycoprotein concentrations after mating are some of the steroid hormones and glycoprotein that may be used to diagnose pregnancies in small ruminants (Refstal et al., 1991). According to Refstal et al. (1991), plasma estrone sulphate may be found in sheep around 70 days after conception and in goats 40-50 days after breeding. A viable pregnancy detection when the estrone sulphate test is positive. Blood P4 levels were considerably lower in ewes whose pregnancies failed in the second half compared to those whose pregnancies were successful (Alexander et al., 2008). A crucial step for strategic management in lowering costs and increasing productive efficiency, early pregnancy detection gives producers the chance to make better-informed choices (Reese et al. 2016). One method for diagnosing pregnancy in animals has been the identification of pregnancy-associated glycoproteins (PAGs), which are aspartic proteases generated in the placenta during pregnancy. This detection is done via laboratory tests like the Enzyme Linked Immunosorbent Assay (ELISA). ELISA assays have shown quicker findings with simple procedures and inexpensive

prices (El Amiri et al., 2007), making them good indicators of pregnancy in domestic animals. PAGs are also detectable by the Idexx Visual Pregnancy Test was found to be effective in detecting PAG as early as the 30th day of pregnancy (Chaves et al., 2017). The fast test that is suggested for use with cows is also useful for detecting pregnancy in sheep and can be detected at day 26 of gestation with accuracy 100% (Chaves et al., 2019). Twenty days after insemination, pregnant sheep may be identified by pregnancy-associated glycoproteins (Karen et al., 2003). The structural similarities between bovine and ovine PAG allow for the detection of ovine PAG using bovine assays, even though commercially available ELISAs employ bovine anti-PAG antibodies for maternal PAG (Rovani et al., 2016).

Materials and Methods

Ethical Approval

Before any experiment performing, the experimental protocol and design used in present study were examined and approved by the Committee of Ethics in College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq (Number P. G/995 at 15/5 2024).

Experimental animals

This study conducted at the farm belong to the College of Veterinary Medicine /University of Baghdad. Eighteen cross adult Iraqi ewes aged between 3-4 years determined by breeding record and verified by dental formula with 3 fertile rams used in this study. The animals subjected to careful clinical examination and ultrasonography examination to determine that they are non-pregnant/healthy and free from diseases. Preventive health measures applied such as vaccination against enterotoxaemia (against clostridia infection) at dose of 2 ml S/C and treatment against internal & external parasites (Rafoxanide and Ivermectin).

Experimental design

The animals were randomly divided into three groups (six ewes each), group A, B and C, group A was received CIDR-PMSG for 12 days (Kadhim and Hussain 2024b), with i/m injection 400 IU of PMSG at withdrawal and group B was received vaginal sponge for 12 days with i/m injection 400 IU of PMSG at withdrawal of the sponges, while group C was not treated and served as control group. The animals kept at semi-opened shade shelter supplemented with drinking water ad libitum, provided with 1kg concentrated diet Balanced diet of grains (barley 40%, wheat 51%, soya been 5%, limestone 2%, NaCl 1%, minerals and vitamins 1%) (Abood, 2012, Abdulkareem et al., 2014, Hussain et al., 2016 and Alrawy and Hussain 2025) daily per ewe. The experiment extend from January 2023 until September 2023.

Blood collection

Blood was collected from jugular vein using vacutainers gel tubes and numbered by hand approximately 5ml, the lower part of the neck of the ewe

was held firmly by the left hand so that jugular vein can be visible. The skin on the jugular vein was cleaned by 70% alcohol. The needle was inserted in jugular vein and collected the blood for determination of PAG and progesterone hormone at the following days of pregnancy 30 and 60. Serum was harvested following centrifugation of samples at 3000 RPM for 10 minutes and then stored at -20°C until the assay (Hussain et al., 2017).

Hormonal study

Determination the concentration of PAG and progesterone during gestation, at following days 30 and 60 of pregnancy. Blood samples were collected at different physiological stages by jugular vein puncture into vacutainer tubes without anticoagulant to test the efficiency of the visual ELISA-PAG in diagnosing pregnancy and non-pregnancy in sheep. The tubes were transported to the laboratory of college veterinary medicine –university of Baghdad in a cool box containing ice bags, centrifuged at 3000 RPM for 10 min, allocated in 1.5ml Eppendorf tubes and stored at -20 C until analysis.

Pregnancy associated glycoproteins were measured using Sheep PAG ELISA KIT and Progesterone were measured using Sheep PROG ELISA KIT. All collected serum samples were frozen and kept at -20°C until assayed. For these assay, in both PAG and P4 added 50 µl in first column of kit plate as standers for test and added 10 µl of (collected serum) and assay controls (positive and negative) were pipetted into 88-well, anti-PAG antibody coated plates along with 40 µl of sample diluent, sealed, and 100µl of conjugate reagent enzyme (anti-IgG-horseradish peroxidase) was added for 96-well with standers and incubated for 60 min at 37°C in a forced air incubator. After incubation, plates were washed four times with 350 µl of wash solution. The detector solution (chromogenic A and chromogenic B) 100 µl was added to each well, covered, and incubated for 15 min at 37°C. The plates were washed (4x) and 100 µl of substrate solution (tetramethylbenzidine) added into the wells and incubated for 15 min at room temperature. Finally, 100 µl of stop solution (foric acid) was added and the absorbance was determined at 450 nm and 630 nm) determined on a microtiter plate spectrophotometer. Sample values were reported as serum sample minus negative controls after subtracting the mean. The interpretation of results was based on the color of the sample at the end of the test. When the pregnancy test was positive, the well was stained blue; in a negative test, well color remained transparent.

Ultrasonographic examinations

Experimental animals were prepared prior examination such as fasting of feed for 12 hrs to obtain the sensitivity, specificity and accuracy of trans rectal and trans abdominal scanning. Sufficient amount of gel was applied to the probe to form good contact and remove air between probe and skin of animal. Animal was examined trans rectal and trans abdominal on standing position. Before trans rectal examination the rectum was evacuated from faces and before trans abdominal examination the

area of abdomen was cleaned with soap and water. Trans rectum examination performed at day 30th after breeding , on average, trans rectal ultrasound examination was performed for diagnosis in each pregnant and nonpregnant sheep, using the liner probe with a 6.5 MHz transducer (de Miranda et al., 2017). After 60 days of breeding all ewes were scanned trans abdominally by using a real-time ultrasound scanner equipped with a 3.5 MHz linear-array transducer (Japan)(Karen et al., 2006). The ewes were scanned in standing position without shaving the ventral abdominal wall. The transducer was applied at the hairless area of the inguinal region of both sides after adding coupling gel (Karen et al., 2006). All ultrasonographic examinations were performed by the same operator. The time spent on each animal to reach a diagnosis was always more than 3 min.

Statistical analysis

Based on the lambing performance, the results of the PAG and progesterone tests were arranged as follows: (a) correct positive diagnosis, (b) incorrect positive

diagnosis, (c) correct negative diagnosis, and (d) incorrect negative diagnosis. From these values, the sensitivity was defined as the ratio between the ewes correctly diagnosed as pregnant and all the pregnant ewes ($a/a + d \times 100$), the specificity was the ratio between the ewes correctly diagnosed as nonpregnant and all the nonpregnant ewes ($c/c + b \times 100$), the positive predictive was the ratio between the ewes correctly diagnosed as pregnant and those diagnosed as pregnant ($a/a + b \times 100$) and the negative predictive value was the ratio between the ewes correctly diagnosed as nonpregnant and those diagnosed as nonpregnant ($c/c + d \times 100$), (Andueza et al., 2014) and the overall accuracy was the ratio between the ewes correctly diagnosed as pregnant and nonpregnant with all the pregnant and nonpregnant (all tested animals), ($a + c/a + b + c + d \times 100$) were calculated (Szenci et al., 1998). The Statistical Analysis System- SAS (2018) program was used to detect the effect of difference factors in study parameters. The three methods of pregnancy diagnosis were calculated but considering PAG method as a gold standard methodology.

Table 1: Equations applying for determining the best method for pregnancy diagnosis in ewes (Kaya et al., 2016 and Khan et al., 2020)

Predictive Value of Positive Test	$100 = 100\% \frac{a}{a+b} \times$
Predictive Value of Negative Test	$100 = 100\% \frac{c}{c+d} \times$
Sensitivity	$100 = 100\% \frac{a}{a+d} \times$
Specificity	$100 = 100\% \frac{c}{b+c} \times$
Accuracy	$100 = 100\% \frac{a+c}{n} \times$

Results and Discussion

Table 2: Pregnancy diagnosis by different methods at day 30 of pregnancy

Animals number	Ultrasonography at day 30 of pregnancy	Pregnancy associated glycoprotein at day 30 of pregnancy	Progesterone at day 30 of pregnancy	Parturition
1	+	+	+	+
2	-	+	+	+
3	+	+	+	+
4	+	+	+	+
5	-	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9	+	+	+	+
10	+	+	+	+
11	+	+	+	+
12	-	+	+	+
13	-	-	-	-

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14	-	-	-	-
15	+	+	+	+
16	-	-	-	-
17	-	-	-	-
18	-	-	-	-
A	10	13	13	
B	0	0	0	
C	5	5	5	
D	3	0	0	
N	18	18	18	

Results of pregnancy diagnosis (positive and negative) by three different methods (ultrasonography scanning, pregnancy associated glycoprotein level and progesterone level)at day 30 of pregnancy for all ewes of study after application different hormonal regimes for estrus synchronization in ewes are presented in (table2). The results proved depending on parturition, by ultrasonography scanning (trans rectal probe 6.5 MHz) the correct positive diagnosis (A) 10 ewes , incorrect positive diagnosis (B) 0 ewe, correct negative diagnosis (C) 5 ewes and incorrect negative diagnosis 3 ewes with total number 18 ewes, while by PAG method show , the correct positive diagnosis (A) 13 ewes , incorrect positive diagnosis (B) 0 ewe, correct negative diagnosis (C) 5 ewes and incorrect

negative diagnosis 0 ewe with total number 18 ewes and by P4 method show , the correct positive diagnosis (A) 13 ewes , incorrect positive diagnosis (B) 0 ewe, correct negative diagnosis (C) 5 ewes and incorrect negative diagnosis 0 ewe with total number 18 ewes. The efficiency of pregnancy associated glycoprotein and progesterone as pregnancy detection tests at day 30 are similar to those previously proved by (Karen, et al., 2003 and Andueza et al., 2014) . In current study there was no evidence of pregnancy losses, since the results of false positive were not detected in P4 and PAG tests at day 30, this results agree with (Andueza et al., 2014). Table 3:Pregnancy diagnosis by different methods at day 60 of pregnancy.

Animals number	Ultrasonography at day 60 of pregnancy	Pregnancy glycoprotein at day 60 of pregnancy	Progesterone at day 60 of pregnancy	Parturition
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9	+	+	+	+
10	+	+	+	+
11	+	+	+	+
12	+	+	+	+
13	-	-	-	-
14	-	-	-	-
15	+	+	+	+
16	-	-	-	-
17	-	-	-	-
18	-	-	-	-

A	13	13	13	
B	0	0	0	
C	5	5	5	
D	0	0	0	
N	18	18	18	

Results of pregnancy diagnosis (positive and negative) by three different methods (ultrasonography scanning, pregnancy associated glycoprotein level and progesterone level)at day 60 of pregnancy for all ewes of study after application different hormonal regimes for estrus synchronization in ewes are presented in (table3). The results proved depending on parturition same results, by ultrasonography scanning (trans abdominal probe 3.5 MHz) the correct positive diagnosis (A) 13 ewes , incorrect positive diagnosis (B) 0 ewe, correct negative diagnosis

(C) 5 ewes and incorrect negative diagnosis 0 ewe with total number 18 ewes, while by PAG method show , the correct positive diagnosis (A) 13 ewes , incorrect positive diagnosis (B) 0 ewe, correct negative diagnosis (C) 5 ewes and incorrect negative diagnosis 0 ewe with total number 18 ewes and by P4 method show , the correct positive diagnosis (A) 13 ewes , incorrect positive diagnosis (B) 0 ewe, correct negative diagnosis (C) 5 ewes and incorrect negative diagnosis 0 ewe with total number 18 ewes.

Table 4:Evaluation of progesterone assay, pregnancy associated glycoprotein and ultrasonography on day 30 of pregnancy by equations as a method for pregnancy diagnosis and comparison between these methods.

	Ultrasonography at day 30 of pregnancy	Pregnancy associated glycoprotein at day 30 of pregnancy	Progesterone at day 30 of pregnancy
Predictive Value of Positive Test	$100=100\%\frac{10}{10}x$	$100=100\%\frac{13}{13}x$	$100=100\%\frac{13}{13}x$
Predictive Value of Negative Test	$100=62.5\%\frac{5}{8}x$	$100=100\%\frac{5}{5}x$	$100=100\%\frac{5}{5}x$
Sensitivity	$100=76.9\%\frac{10}{13}x$	$100=100\%\frac{13}{13}x$	$100=100\%\frac{13}{13}x$
Specificity	$100=100\%\frac{5}{5}x$	$100=100\%\frac{5}{5}x$	$100=100\%\frac{5}{5}x$
Accuracy	$100=83.3\%\frac{15}{18}x$	$100=100\%\frac{18}{18}x$	$100=100\%\frac{18}{18}x$

Results of pregnancy diagnosis (predictive value of positive test, predictive value of negative test, sensitivity, specificity and accuracy) by three different methods (ultrasonography scanning, pregnancy associated glycoprotein level and progesterone level)at day 30 of pregnancy for all ewes of study after application different hormonal regimes for estrus synchronization in ewes are presented in (table4). The results proved depending on parturition, by ultrasonography scanning (trans rectal probe 6.5 MHz) predictive value of positive test, predictive value of negative test, sensitivity, specificity and accuracy is (100%, 62.5%, 76.9%, 100% and 83.3% respectively) with total number 18 ewes, while by PAG method show , predictive value of positive test, predictive value of negative test, sensitivity, specificity and accuracy is (100%,

100%, 100%, 100% and 100% respectively) with total number 18 ewes and by P4 method show, predictive value of positive test, predictive value of negative test, sensitivity, specificity and accuracy is (100%, 100%, 100%, 100% and 100% respectively) with total number 18 ewes.

The important of using two tests (the PAG test over the P4 test) is that it possible to distinguish between pregnancy and prolonged inter estrus intervals, this is supported by Karen et al. (2003). Our results agree with de Miranda et al.,(2017) , who demonstrated that ELISA-PAG test may be considered as an alternative for rapid and accurate detection of PAG, as well as the identification non-pregnant ewes and it showed similar or better sensitivity as trans-rectal ultrasonography for pregnancy detection. Although present study show the same results

of sensitivity and specificity and accuracy between progesterone and pregnancy associated glycoprotein at day 30 of pregnancy, it agrees with Karen , et al.,(2003) and Boscos et al., (2003) who show that the PAG-RIA test had significantly higher specificity for diagnosing non-pregnant ewes than P4 test ,because it may be attributable to fact that PAG is produced by trophoblastic binucleate cells, but P4 level attributable to functional corpus luteum that is associated with gestation, ovarian or uterine pathology(hydrometra or pyometra) or normal estrus cycle. Therefor, this is an important limitation of progesterone tests for detection of pregnancy. Also Karen et al., (2003) show the accuracy of pregnancy diagnosis in PAG test higher than those reported for ultrasonography during the first 30 days of pregnancy, As well as show the sensitivity and specificity of PAG tests at day 22 of pregnancy were high (93.5 and 100% respectively) and similar results detected by Gonzalez et al., (1999) in goat. On the other hand Ranilla et al.,(1994) show sensitivity of PAG is only 66.6% in Merino and Churra breed of sheep at day 21 of pregnancy which lower than our results . The serum PAG-ELISA in our study being investigated here revealed that this technique is confirmed and reliable to be used early in pregnancy detection in sheep. It matured as a reliable method after 18 days and it achieved 100%.Also the results of ELISA-PAG shown that it was an efficient, rapid, and suitable method for early pregnancy diagnosis in the sheep and it constituted a tool to reproductive biotechnologies ranging used from the 30th day of gestation in ewes It therefore provides pregnancy determination comparable with other modern pregnancy diagnosis approaches, this result is approximately like the results found by El Amiri et al., (2014) that PAG-ELISA reliable with 100% at day 24 of pregnancy and other studies RIA, provided the sensitivity of 100% from day 29 of pregnancy and specificity of 100% of day 22 of pregnancy (Karen et al. 2003). In contrary, ELISA tests are more practical. The ELISA method which was used was confounded by the antibody and types the blood samples used which led to reported sensitivity to range from 93.5 % on day 23 (Rovani et al. 2016) to 100 % on day 24 post mating (El Amiri et all. 2014) and the specificity was reported as 98.9% in varying time of pregnancy (Rovani et al. 2016). Also reported that sensitivity, specificity and

accuracy by Khan et al., (2020) is 95.23% ,91.67% and 96.66% respectively in sheep and 100%, 100% and 100% respectively in goat Detection of Pregnancy associated glycoprotein (PAG) results in about 95.3% of early expected pregnancy confirmation as early as Day 18 of pregnancy (Barbato et al., 2009). A new color-binding polyclonal antibody based enzyme-linked immuno sorbent assay kit (CER-6900; Marloie) was tested for pregnancy diagnosis in the Rasa Aragonesa breed, exhibiting sensitivity and specificity of 100% on Day 25 and forward (Alabart et al., 2010). Chaves et al. (2019) found that rapid PAG test recommended for cow is efficient for pregnancy diagnosis in sheep with efficiency of 100% at day 26 of pregnancy. The important of PAG test is due to demand single sample for early detection of pregnancy, also the accuracy of this test for pregnancy detection is higher than those detection by ultrasonography during first 30 days of pregnancy which also proved by Karen et al., 2003). Also Ganaie et al. (2009) detected progesterone in serum ewes with accuracy 98% between day 15 and 30 of pregnancy. Our results also in agreement with De Carolis et al., (2020) who proved that P4 and PAG values can early detect and distinguish between pregnant and non pregnant ewes with sensitivity , specificity and accuracy are 100%. Also Babato et al., (2009) proved as early as that sensitivity, specificity , PPV and NPV for progesterone test were 100% therefor this test can be a reliable marker for pregnancy detection at this period. In support of our observation Yotov, (2007) showed that detection of P4 level was more reliable than echography in order to pregnancy diagnosis at the first 30 day of pregnancy. In support of our study Roberts et al. (2019) found that sensitivity and specificity of ultrasonography test at day 30 after mating was 83 and 100% respectively. Anwar et al. (2008) in agreement with present study which detected the accuracy of pregnancy diagnosis by ultrasonography was 80% at day 31-35 and 100% at day 42 of pregnancy. Also Ganaie et al. (2009) and Tasal et al. (2006) detect pregnancy by ultrasonography with accuracy 79.7% between day 30 and 45 of pregnancy in Akkraman and Awassi sheep. Garcial et al. (1993) detected pregnancy by ultrasonography at day 32 of pregnancy with accuracy 85%, these results close to our results.

Table 5:Evaluation of progesterone assay, pregnancy associated glycoprotein and ultrasonography on day 60 of pregnancy by equation as a method for pregnancy diagnosis and comparison between these methods

	Ultrasonography at day 60 of pregnancy	Pregnancy associated glycoprotein at day 60 of pregnancy	Progesterone at day 60 of pregnancy
Predictive Value of Positive Test	$100=100\%\frac{13}{13}x$	$100=100\%\frac{13}{13}x$	$100=100\%\frac{13}{13}x$
Predictive Value of Negative Test	$100=100\%\frac{5}{5}x$	$100=100\%\frac{5}{5}x$	$100=100\%\frac{5}{5}x$

Sensitivity	$100=100\% \frac{13}{13} x$	$100=100\% \frac{13}{13} x$	$100=100\% \frac{13}{13} x$
Specificity	$100=100\% \frac{5}{5} x$	$100=100\% \frac{5}{5} x$	$100=100\% \frac{5}{5} x$
Accuracy	$100=100\% \frac{18}{18} x$	$100=100\% \frac{18}{18} x$	$100=100\% \frac{18}{18} x$

Results of pregnancy diagnosis (predictive value of positive test, predictive value of negative test, sensitivity, specificity and accuracy) by three different methods (ultrasonography scanning, pregnancy associated glycoprotein level and progesterone level)at day 30 of pregnancy for all ewes of study after application different hormonal regimes for estrus synchronization in ewes are presented in (table5). The results proved depending on parturition, by ultrasonography scanning (trans abdominal probe 3.5 MHz) predictive value of positive test, predictive value of negative test, sensitivity, specificity and accuracy is (100%, 100%, 100%, 100% and 100% respectively) with total number 18 ewes, also by PAG method show , predictive value of positive test, predictive value of negative test, sensitivity, specificity and accuracy is (100%, 100%, 100%, 100% and 100% respectively) with total number 18 ewes and by P4 method show, predictive value of positive test, predictive value of negative test, sensitivity, specificity and accuracy is (100%, 100%, 100%, 100% and 100% respectively) with total number 18 ewes. Although the current results is same in three methods, PAG is alternative methods for pregnancy diagnosis because is easier faster and evaluated greater number of ewes than other methods.

Our results in agreement with De Carolis et al., (2020) who proved that P4 and PAG values can detect and distinguish between pregnant and non pregnant ewes at day 60 with sensitivity , specificity and accuracy 100% . In support of our observation Yotov, (2007) showed that higher percentage of positive predicted values by the progesterone test at day 60 of pregnancy, even though did not observe any variation between ultrasonography and P4 tests in negative predictive values and accuracy for these parameters of P4 at day 60 and echography on day 40 of pregnancy were close to results by Karen et al., (2006) . Anwar et al. (2008) in agreement with present study which observed Placentome and leg in 100% ewes between day 45 and 50 of pregnancy also observed vertebral column with 100% cases between day 51 and 55 of pregnancy. Ganaie et al. (2009) detect pregnancy by transabdominal ultrasonography with accuracy 100% between day 60 and 75 and Tasal et al. (2006) detect pregnancy by transabdominal ultrasonography with accuracy 98.8% between day 60 and 80 of pregnancy in Akkraman and Awassi sheep. Also Kaulfuss et al. (1996) detected pregnancy by ultrasonography with accuracy 100% at day 60 of pregnancy in German Merino sheep. It can be concluded that ovine pregnancy is reliably diagnosed at day 30 after mating by using sheep PAG-

ELISA and sheep P4- ELISA, but PAG- ELISA consider the most reliable because to P4 from CL of pregnancy or from CL of other physiological or pathological sources.

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