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Pregnancy Diagnosis Methods in Cows

İneklerde Gebelik Tanı Yöntemleri

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ABSTRACT

In cattle farms, optimising milk yield and fertility is crucial in terms of sustainability and economics. In order to optimise these parameters, each cow should have a calf every 12-14 months. For this purpose, cows should be pregnant during the earliest postpartum period and undergo pregnancy examinations promptly. Early diagnosis of pregnancy on cattle farms is essential for reproductive management. Methods of pregnancy diagnosis are divided into two groups, direct and indirect. Direct diagnostic methods objectively determine the state of pregnancy. Indirect diagnostic methods provide information about pregnancy through biomarkers formed in the maternal circulation during pregnancy. An ideal pregnancy test should be able to give accurate results in early pregnancy (high sensitivity and specificity) and should be low cost and easy to apply. Although direct diagnostic methods are the most commonly used today, it is predicted that the frequency of use of indirect diagnostic methods will increase and new methods will be developed as a result of developing technology and progress in scientific studies. The present review aims to inform the readers about the methods that can be used in the diagnosis of pregnancy in cows, how these methods are performed, their advantages and disadvantages compared to each other.

Keywords: Cow, Direct diagnosis, Indirect diagnosis, Pregnancy diagnosis

ÖZ

Büyükbaş hayvan çiftliklerinde süt veriminin ve doğurganlığın optimize edilmesi sürdürülebilirlik ve ekonomi açısından büyük önem taşımaktadır. Bu parametreleri optimize etmek için her ineğin 12-14 ayda bir buzağılaması gerekmektedir. Bu amaçla ineklerin postpartum en erken dönemde gebe kalmaları ve derhal gebelik muayenelerinden geçmeleri gerekmektedir. Sığır çiftliklerinde gebeliğin erken teşhisi üreme yönetimi açısından önemlidir. Gebelik tanı yöntemleri direkt ve indirekt olmak üzere iki gruba ayrılmaktadır. Direkt tanı yöntemleri gebeliğin durumunu objektif olarak belirler. İndirekt tanı yöntemleri ise gebelik sırasında anne dolaşımında oluşan biyobelirteçler aracılığıyla gebelik hakkında bilgi sağlamaktadır. İdeal bir gebelik testi erken gebelikte doğru sonuçlar verebilmeli (duyarlılığı ve özgüllüğü yüksek), maliyeti düşük ve uygulaması kolay olmalıdır. Günümüzde gebelik tanısı amacıyla çoğunlukla direkt tanı yöntemleri kullanılmakla birlikte, gelişen teknoloji ve bilimsel çalışmalardaki ilerleme sonucunda indirekt tanı yöntemlerinin kullanım sıklığının artacağı ve yeni yöntemlerin geliştirileceği öngörülmektedir. Bu derlemenin amacı, ineklerde gebelik teşhisinde kullanılabilecek yöntemler, bu yöntemlerin nasıl uygulandığı, birbirlerine göre avantaj ve dezavantajları hakkında okuyucuları bilgilendirmektir.

Anahtar Kelimeler: İnek, Direkt tanı, Gebelik tanısı, İndirekt tanı

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INTRODUCTION

The primary aim of cattle breeding is to optimise milk production and fertility by calving cows at 12-14 months intervals. In order to achieve this objective, cows should become pregnant again between 85 and 145 days after calving (Fetrow et al., 2007). In cattle farms, it is of crucial importance to ensure that a cow becomes pregnant as soon as possible after the voluntary waiting period has passed and to perform pregnancy examination at the earliest period. Given that the annual care and feeding costs for pregnant and non-pregnant cows are similar, retaining a non-pregnant cow in the enterprise may lead to economic disadvantages. Early diagnosis of pregnancy in cows is a crucial part of productivity and sustainability, especially in dairy farms (Balhara et al., 2013, Pohler et al., 2016).

Early pregnancy diagnosis is crucial for the early identification of non-pregnant (open) animals in an enterprise and the treatment or removal of these animals from the herd, if necessary, without losing time. The ideal pregnancy test should have high sensitivity, the ability to accurately identify pregnant animals, and high specificity, the ability to accurately identify non-pregnant animals. In addition, an ideal pregnancy test should be practical and cost-effective so that it can be applied under field conditions. Pregnancy diagnosis methods are divided into two as direct and indirect methods (Figure 1.). While direct methods allow pregnancy to be felt or seen objectively, indirect methods use some biomarkers to learn pregnancy status (Fricke et al., 2016). Direct methods require special tools and equipment and an experienced veterinarian, whereas indirect methods require special laboratories and some tests (Akköse and Çiğdem, 2019).

The aim of this review is to provide information to the reader about the pregnancy diagnosis methods that can be used in cows today, how these methods are performed, and the advantages and disadvantages of these methods compared with each other.

Pregnancy Diagnosis Methods

Direct diagnosis methods

Direct diagnostic methods are non-subjective methods for detecting pregnancy that rely on objective signs or visual confirmation. When applying these methods, an experienced veterinarian and special tools and equipment are needed (Balhara et al., 2013).

Oestrus following after fertilisation

The lack of oestrus signs in a cow or heifer approximately 18-24 days after fertilisation may indicate the presence of a pregnancy. In this pregnancy diagnosis method, it is significant that oestrus observation is performed carefully and accurately. In addition, this diagnostic method may give false positive results in cases such as anoestrus, suboestrus, persistent corpus luteum because not all cattle have a regular oestrus cycle (Sheldon and Noakes, 2002). While it is generally observed that pregnant animals do not show signs of estrus, approximately 6% of pregnant cattle may exhibit such signs. These symptoms are observed at all stages of pregnancy and mostly in the 4th-8th months (Kumar et al., 2014).

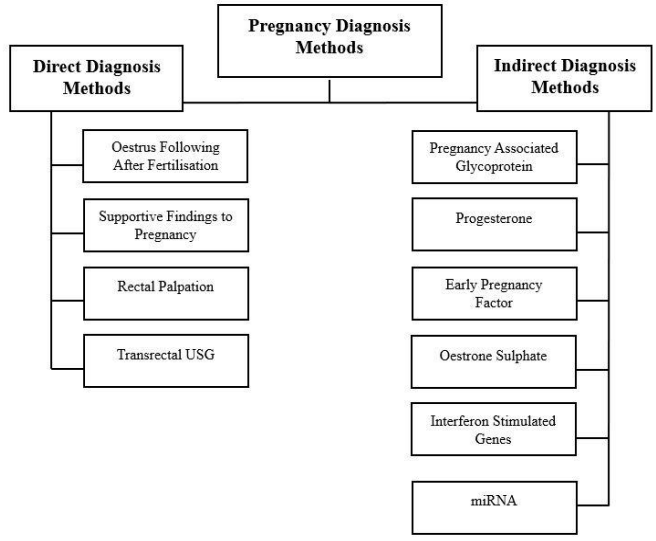

Figure 1. *Pregnancy Diagnosis Methods*
Şekil 1*. Gebelik Tanı Yöntemleri*

Supportive findings to pregnancy

- *Persistence of the corpus luteum*

The corpus luteum produces progesterone which is necessary for pregnancy. The presence of an active corpus luteum in the ovary of a cow at 3-4 weeks after artificial or natural insemination indicates that pregnancy is possible (Utt et al., 2009). In non-pregnant cows, persistent corpus luteum and the presence of corpus luteum in prolonged diestrus are also observed (Siqueira et al., 2019).

- *Asymmetry in cornus*

During pregnancy, the cornu uteri, in which pregnancy occurs, grows massively due to the accumulation of allantoyic fluid in the chorioallantoyic membrane and asymmetry occurs between the cornus due to this growth. As a result of the growth of the uterus, thinning of the uterine layers and decrease in tubular structure are observed. However, this asymmetry between the cornu can also be caused by multiple births, delayed uterine involution, uterine pathologies and fluid accumulation in the uterus (Sheldon and Noakes, 2002).

- *Feeling the fremitus*

In order to provide the increased nutritional needs of the fetus during pregnancy, 'Fremitus' is formed as a result of hypertrophy of the Arteria Uterina Media and increased blood flow. Fremitus can be felt unilaterally from the third month of pregnancy and bilaterally from the 5th month of pregnancy in pregnant animals (Christiansen, 2014).

Rectal palpation

Rectal palpation, which has been used in veterinary medicine for many years, is a method routinely used today. In this diagnostic method, pregnancy status is decided by palpation of the structures of pregnancy (Jaskowski et al., 2019).

- *Palpation of chorioallantoyis*

Chorioallantois can be palpated in cows between days 35-40 of gestation. During rectal palpation, it is possible to feel the membranes slipping, but the membranes can also be palpated as a result of embryonic-fetal death (Jaskowski et al., 2019).

- *Palpation of the amniotic sac*

The amniotic sac is detectable as a swelling containing fluid, similar to a distended balloon, and can be palpated between days 28 to 35 of gestation in cows. After the day 65 of pregnancy, the amniotic sac can no longer be palpated as it loses its swelling with the growth of the fetus (Sheldon and Noakes, 2002).

- *Palpation of placentomes*

Placentomas start to form approximately in the first month of pregnancy. However, they reach a size that can be felt by rectal palpation in approximately the third month of pregnancy (Jaskowski et al., 2019).

- *Palpation of the fetus*

From approximately the second month of pregnancy onwards, the fetus can be palpated as the amniotic sac loses its swelling (Romano et al., 2007).

Transrectal Ultrasonography

Transrectal Ultrasonography (USG) has been routinely used for pregnancy examination in cows since the 1980s. Ultrasound probes of 3.5 – 5 – 7.5 megahertz (MHz) are generally preferred in cows. While 3 MHz probes are used for maximum penetration, 7.5 MHz probes are used for better resolution. In a study (Hanzen and Delsaux, 1987) which a 3 MHz probe was used for early pregnancy examination in cows, it was reported that the pregnancy confirmation rate was 94% on the 25 day after insemination, while in another study (Boyd et al., 1990) which a 7.5 MHz probe was used, it was reported that this rate was 100% on the 20 day after insemination. There are 3 types of ultrasonography methods used for pregnancy examination in cows.

- *A-Mode USG (Amplitude Mode)*

In A-Mode USG, an one-dimensional image is formed on the screen. Ultrasound waves produced as a result of the vibration of the crystals in the probe form a linear graphic due to the reflections from the structures it encounters. A-Mode USG is not used much today because of its low pregnancy detection rate and its inability to determine the number and viability of fetuses (Ganaie et al., 2009).

- *Realt Time B-Mod USG (Brightness Mode)*

It is known that pregnancy diagnosis by Real Time B-Mod Ultrasonography in cows was first performed in 1982. With the widespread use in veterinary medicine over time, it is now used as a routine pregnancy examination method. Linear probes are generally used in cows. The sound waves produced by these probes create images in different shades of white-gray-black colours on a black background according to the structure of the target tissue. With this method, it is also possible to see the movements of the examined structure (Sheldon and Noakes, 2002).

B-Mode USG is the most commonly used type of ultrasound for the diagnosis of pregnancy in cows because of its high accuracy, ease of application, rapid results, easy accessibility and information about the fetus (viability, age, sex and number) (Akköse and Çiğdem, 2019).

In a study (Romano et al., 2006) on ultrasonographic examination in cows, pregnancy was diagnosed between 16 and 23 days after fertilization. Although it states that it can give accurate results in days 26-30 after fertilization, the highest accuracy is achieved. It is stated that it can be reached on days.

- *D-Mod USG (Doppler Mode)*

In Doppler Mode USG, the sound waves emitted from the probe create colours according to the direction of blood flow in the tissue it will encounter (blood coming towards the probe= red colour; blood moving away from the probe= blue colur). The brightness of the coloured pixels indicates the amplitude of the frequency difference. As the frequency difference increases, the pixel brightness also increases. Fetal heartbeat and fetal number can be determined by Doppler USG. Therefore, it is used more frequently than A-Mode USG (Ginther, 2007).

Indirect diagnosis methods

These are methods which are not directly related to pregnancy, but in which some biomarkers which occur as a result of pregnancy and are found in maternal body fluids are used. These are generally hormonal and molecular methods and require special laboratory conditions (Fricke et al., 2016).

Pregnancy associated glycoprotein (PAG)

PAG derivatives in ruminant species consist of a large family. Pregnancy-associated glycoproteins are analysed in two groups as 'Ancient' and 'Modern' (Garbayo et al., 2008). There are mononuclear and binuclear cells in the chorion epithelium of ruminants. With the differentiation of mononuclear cells, binuclear cells are formed. Binuclear cells transfer the secretion granules they form to the maternal plasma. These secretions constitute the majority of PAGs (Green et al., 2000).

There are 22 PAG gene families in cows and the first one is Pregnancy Specific Protein-B (PSPB). Protein-B is generally

not preferred because its concentration in maternal plasma is lower than other PAG derivatives and its half-life is longer (Green et al., 2000). In mammals, there are some special molecules (eCG in mares, hCG in humans) that form temporary connections between mother and offspring. In cattle, these molecules are PAGs produced in the villi chorialis of the embryo (Telugu et al., 2009).

Pregnancy-associated glycoproteins are used in the diagnosis of embryonic/fetal deaths and twin pregnancies as well as pregnancy diagnosis. Factors such as milk yield, gestation period, number of lactations, breed, foetal sex, calf birth weight, maternal weight, twinning, heat stress and postpartum illness affect serum and milk PAG levels (Serrano et al., 2009).

PAG tests are generally used on days 26-30 after fertilisation for the diagnosis of pregnancy in cows (Fricke et al., 2016). Pregnancy-associated glycoproteins can be analysed from maternal plasma, serum, milk or whole blood samples by PAG-ELISA methods (Gábor et al., 2007).

Progesterone (P4)

Progesterone is a steroid hormone released during the dioestrus phase of each oestrus cycle after puberty and is essential for the maintenance of pregnancy. Progesterone was first isolated from corpus luteum extract in 1934 and is known to be structurally derived from cholesterol (Alaçam, 2015).

Natural P4 is synthesised and secreted from luteal cells in the corpus luteum during the luteal phase of the cycle and from both the corpus luteum and ovary during pregnancy, with species-specific differences. In addition, progesterone is also secreted from ovarian follicles, testes and adrenal gland, which are the site of production of some steroid hormones such as estrogen, androgen, testosterone and corticosteroids (Ergene, 2008).

Conceptus inhibits the luteolytic mechanism, maintaining the corpus luteum and thus the production of progesterone. Progesterone makes the uterine endometrium suitable for implantation and maintenance of pregnancy. Progesterone concentration varies according to the stage of the oestrus cycle. In this way, it is used in the diagnosis of pregnancy in cows and also in the diagnosis of ovarian cysts (Mimoune et al., 2021).

In cows, high P4 concentration ($\geq$ 2 ng/ml) at 18-24 days after fertilisation is used to diagnose pregnancy, but this method is not always accurate. This is due to progesterone concentrations determined as a result of prolonged dioestrus, luteal cysts, pyometra and inaccurate recording of insemination time (Vural et al., 2012).

Since the progesterone concentration in non-pregnant cows is always $\leq$ 1 ng/ml after 18-24 days post-fertilisation, it is more accurate to identify non-pregnant animals when examining pregnancy with progesterone (Alaçam, 2015).

Progesterone measurement for pregnancy diagnosis in cows can be performed by qualitative (colour) and quantitative (quantity) methods in milk and blood samples. It is reported that the accuracy rate of pregnancy examination with milk and blood samples is similar (Otavă et al., 2007).

Early pregnancy factor (EPF)

Early pregnancy factor is a glycoprotein with a molecular weight of 10.84 kDa, first isolated from pregnant mice (Cavanagh, 1996). Early pregnancy factor is a biomarker that can be used for the diagnosis of pregnancy in humans (Smart et al., 1982), sheep (Morton et al., 1979), cattle (Nancarrow et al., 1981), pigs (Grewal et al., 1985), mares (Ohnuma et al., 1996), deer (Lash et al., 1997) and marsupial rats (Cruz et al., 2001). Early pregnancy factor is produced by maternal tissues in response to the presence of conceptus after successful fertilisation and continues to be produced by the embryo after implantation. In order for EPF to be produced in maternal plasma, there must be a viable conceptus that has reached the blastocyst stage. Early pregnancy factor is a biomarker that exerts an immunomodulatory effect on maternal acceptance of the zygote, which is defined antigenically as a foreign substance to the body. Early pregnancy factor has two different components, EPF-A and EPF-B. Early pregnancy factor-A is secreted from the oviduct, while EPF-B is secreted from the ovary (Youngquist, 2006).

Early pregnancy factor is produced in the 6-24 hours after fertilisation and is absent in maternal plasma in the 24-48 hours after conceptus death (Balhara et al., 2013). The rapidly decreasing EPF concentration in the maternal circulation as a result of embryonic/fetal death reduces the possibility of false positive results of this test and thus makes it an ideal pregnancy test (Youngquist, 2006). However, because of the high rate of early embryonic death (28-43%) (Diskin et al., 2006) and the increase in plasma EGF concentration due to tumours and non-placental sources, a repeat pregnancy examination should be performed at a later date.

Rosette Inhibition Test (RIT) can be used to determine the presence of EGF for pregnancy diagnosis. Although the RIT is a highly accurate test, it is not generally used in field conditions due to the long application time and the difficulties encountered during the application phase. A test kit has been developed to determine EPF under field conditions, but the accuracy of this test is reported to be low (Baştan et al., 2007).

Oestrone sulphate

Oestrone sulphate is a hormone conjugated to oestradiol with the enzyme oestrogen sulphotransferase and produced in the foetal/cotyledonary portion of the placenta. It is therefore recognised as a marker of pregnancy in cows. Oestrone sulphate is usually present in foetal fluids (amnion-allantoyis) and maternal plasma (Shah et al., 2006).

Since estrone sulphate concentration cannot be determined reliably until the day 80 of pregnancy, the ideal test time is considered to be the 80-100 day after fertilisation (Balhara et al., 2013). The inability to determine maternal

oestrone sulphate concentration in early pregnancy causes this test to be less preferred than other pregnancy tests.

The amount of oestrone sulphate in the maternal circulation can be measured by RIA-EIA methods in blood and milk samples (Isobe et al., 2003). Many factors such as litter size, genetic structure, maternal body weight and environmental factors affect the concentration of oestrone sulphate in maternal plasma (Lobago et al., 2009).

Interferon stimulated genes (ISGs)

Interferon-tau (IFN-τ) is a biomarker of pregnancy in cattle that ensures maternal recognition of pregnancy and the absence of an antigenic response in maternal plasma. IFN-τ secreted by trophoectoderm cells of the conceptus acts paracrinally on the uterine endometrium, inhibiting the pulsatile release of prostaglandin F2α. In this way, IFN-τ prevents the formation of a luteolysis mechanism and ensures the successful continuation of pregnancy (Hansen et al., 2017).

Interferon-tau causes an increase in the concentration of Interferon Stimulated Genes (ISGs) on maternal leukocytes. Today, it is known that there are more than 100 ISGs and among them Myxovirus Resistance Gene 1-2 (Mx 1-2), ISG15, 2'5' Oligoadenylate Synthetase (OAS1) are widely used in pregnancy diagnosis (Green et al., 2010).

Interferon Stimulated Genes on maternal leucocytes are detected approximately on the day 14 of pregnancy and reach a peak level on the 17-18 days. In a study (Gifford et al., 2007), it was reported that Mx-2, ISG15 and Mx-1 concentrations in peripheral blood leucocytes increased on days 16, 18 and 20 of pregnancy, respectively. The same study states that ISG15 and Mx-2 genes give more reliable results in pregnancy diagnosis. However, the increase in the concentration of genes such as ISG15 as a result of viral infections limits the reliability of these tests (Palomares et al., 2013).

RT-PCR method can be used in blood samples for the measurement of ISG concentration for pregnancy diagnosis in cattle (de Melo et al., 2020). Since it cannot be performed easily under field conditions and requires special laboratory conditions, its use in pregnancy diagnosis is less common than other tests.

miRNA

MicroRNAs (miRNAs) are molecules which are 18-22 nucleotides long, play a role in the regulation of gene expression and can be found in most biological fluids including amniotic fluid, serum, urine and milk (Pohler et al., 2015). miRNAs are secreted from extracellular vesicles attached to the plasma membrane and especially from exosomes. Extracellular vesicles are small structures released by almost every cell. The exosome complex is a multi-protein compound involved in the degradation of various RNAs. Exosomes (40-100 nm) and microvesicles (50-1000 nm) are the most common types of extracellular vesicles involved in the control of various reproductive processes, including capacitation, fertilisation and embryo-endometrial attachment (Kurian and Modi, 2019).

In a study on miRNAs (Fiandanese et al., 2016), it was reported that 'bta-mir140', a miRNA derivative, could be used as a pregnancy marker in cattle and the concentration of bta-mir140 in maternal plasma increased significantly on the day 13 of pregnancy.

In another study (Ioannidis and Donadeu, 2016), conducted with cattle, it was reported that 6 different miRNA derivatives increased on certain days of pregnancy. It was reported that 'bta-miR-26a, bta-miR-29c, bta-miR-138, bta-miR-204' increased on the day 16 of pregnancy, 'bta-miR-1249' increased on the day 24 of pregnancy and 'hsa-miR-4532' increased on the days 16-24 of pregnancy. In addition, a human study (Gilad et al., 2008) reported the presence of miRNAs that increased in pregnant women but not in non-pregnant women. Detection of miRNA presence for pregnancy diagnosis in cows is a reliable method, but it is less used than other pregnancy diagnosis methods due to the special laboratory conditions required and high cost.

CONCLUSION

As a result; early diagnosis of pregnancies in the enterprise reduces the cost of care of non-pregnant cows. There are many methods for early diagnosis of pregnancy in cows. None of the pregnancy diagnosis methods; it is not considered an ideal diagnostic method due to accuracy limitations and the need for special laboratory and equipment. Currently, rectal palpation and transrectal ultrasonography are commonly used methods to determine cow pregnancy at an early stage due to their low cost, easily applicable, accurate and rapid results. Due to embryonic deaths that may occur in the early stages of pregnancy, the pregnancy status of cows should be confirmed with the PAG test, which can provide rapid and highly accurate results, one week after the pregnancy examination by USG or rectal palpation. Although direct diagnostic methods are mostly used today, it is predicted that indirect methods will be used more frequently thanks to the developing technology and progress in science.

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