# Pregnancy Diagnosis in Dairy Cows

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dairy cow        ultrasonography        pregnancy proteins        embryonic mortality        fetal mortality

# 1. Introduction

The extent of pregnancy loss (PL) in dairy cows can be estimated from the difference between the fertilization rate (FR) and the subsequent calving rate (CR). Therefore, 41% to 57% of pregnancies can be lost during gestation. Most of the losses (early embryonic mortality: EEM) may occur before the maternal recognition of gestation [1]. After the maternal recognition of pregnancy, the life of the corpus luteum will be extended, and embryonic mortality (further 5% to 10%) occurring between Days 16 and 42 after AI is called late embryonic mortality (LEM), while occurring between Days 42 to 90 after AI is named early fetal mortality (EFM).

Recently, Pohler et al. [2] have suggested using the terms 'early embryonic development' until Day 30 and 'late embryonic development' between Days 31 and 45 of gestation. Due to the fact that different technical terms are used for this period, perhaps it would be easier to divide the former late embryonic period between Days 16 to 42 into two stages, namely, stage 1 between Days 16 and 30 and stage 2 between Days 31 and 42/45 of gestation.

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# 2. Early Pregnancy Diagnosis (PD)

During ultrasonographic examination (UE), a cow was initially considered to be pregnant when an irregularly shaped, non-echogenic black spot (or spots) were recognized within the uterine lumen, representing the chorioallantoic fluid [3]. Demonstration of an embryo and/or embryonic membranes provided additional confirmation of pregnancy [4][5]. The confirmation of ultrasonographic diagnoses was usually based on palpation per rectum of the uterus at 2 to 3 months post-AI, or upon spontaneous return to estrus after AI. A cow was also considered pregnant if an embryo proper with a beating heart was recognized at a final UE on Days 50 to 60 post-AI.

Under experimental conditions, embryonic vesicles in dairy cows can be detected as early as at 9 [6], 10 [7], or 12 days [8] of gestation; however, an accurate pregnancy diagnosis (100%) can be reached only on Days 20 and 22 of gestation [9], when the embryo (20.3 days) and its heartbeat (20.9 days) can be detected [10]. It is important to mention that fluid accumulation in the chorioallantois during early pregnancy can be easily confused with the uterine fluid within the uterus during pro-estrus and estrus [9].

Under on-farm conditions, between Days 22 to 49 after AI, the sensitivity, specificity, positive predictive value, and negative predictive value of ultrasonographic diagnoses made with a 5 MHz linear-array or sector transducer varied between 95% and 100%, 67% and 100%, 85.7% and 100%, and 84% and 100%, respectively (Table 1).

**Table 1.** Accuracy of ultrasonographic examinations for diagnosing early pregnancy in dairy cattle by using a 5.0 MHz linear-array or sector transducer.

| Days after AI | N | Sensitivity (%) | Specificity (%) | Positive Predictive Value (%) | Negative Predictive value (%) | References |
|---|---|---|---|---|---|---|
| 22–40 | 435 | 96.2 | 71.1 | 89.6 | 87.8 | Filteau and DesCoteaux, [11] |
| 25–29 | 101 | 98 | 91.3 | 93 | 97.6 | Szenci et al. [12] a |
| 30–39 | 143 | 100 | 100 | 100 | 100 | |
| 26 b | 48 | 100 | 96.7 | 94.4 | 100 | Romano et al. [5] |
| 27 b | 53 | 100 | 92.8 | 92.6 | 100 | |
| 26–33 | 85 | 97.7 | 87.8 | 89.6 | 97.2 | Pieterse et al. [3] |
| 29 c | 151 | 100 | 96.3 | 91.3 | 100 | Romano et al. [5] |

| Days after AI | N | Sensitivity (%) | Specificity (%) | Positive Predictive Value (%) | Negative Predictive value (%) | References |
|---|---|---|---|---|---|---|
| 30 [c] | 151 | 100 | 97.4 | 91.9 | 100 | |
| 30–39 | 722 | 95 | 67 | 89 | 84 | Hanzen and Laurent [13] |
| 40–49 | 620 | 98 | 77 | 92 | 94 | |
| 31–35 | 323 | 98 | 80 | 85.7 | 97 | Munoz del Real et al. [14] |

[a] Sector transducer, [b] Heifers, [c] Cows, NG: not given. Sensitivity = correct positive/correct positive + false negative × 100. Specificity = correct negative/correct negative + false positive × 100. Positive predictive value = correct positive/correct positive + false positive × 100. Negative predictive value = correct negative/correct negative + false negative × 100.

In contrast, Badtram et al. [15] reported that the sensitivity and specificity of the ultrasound test between Days 23 and 31 post-AI were only 68.8% and 71.7%, respectively. In a recent study, maximum sensitivity and negative predictive value were reached at Day 26 in dairy heifers and at Day 29 in dairy cows, while the specificity and positive predictive values were 96.7% and 94.4% for dairy heifers and 96.3% and 91.3% for dairy cows, respectively [5]. In contrast, according to Nation et al. [4], due to pregnancy loss, Days 28 to 35 after AI are too early for reliable detection of pregnancy in dairy cows.

Between Days 27 to 34 after AI, the sensitivity, specificity, positive predictive value, and negative predictive value of ultrasonographic diagnoses made by the use of 5–10 MHz transducer varied between 90% and 96.6%, 91.5% and 100%, 88.4% and 100%, and 92.3% and 97.8%, respectively (Table 2). When the recognition of an embryo proper with a beating heart was used as the criterion for a positive ultrasound PD, significantly (p < 0.001) more false negative and less false positive ultrasound diagnoses were made, in comparison with the recognition of chorioallantoic fluid [16]. In contrast, Silva et al. [17] reported that the presence of chorioallantoic fluid in the uterine

lumen and a corpus luteum alone might lead to more false positive results than when an embryo was visualized by using a 5–10 MHz linear-array transducer on Day 27 after timed AI.

**Table 2.** Accuracy of ultrasonographic examinations for diagnosing early pregnancy in dairy cattle by using a high frequency linear-array transducer.

| Days after AI | N | Sensitivity (%) | Specificity (%) | Positive Predictive Value (%) | Negative Predictive Value (%) | References |
|---|---|---|---|---|---|---|
| 27 | 1673 | 96.5 | 93.4 | 89.7 | 97.8 | Silva et al. [17] (5–10 MHz transducer) |
| 28 | 100 | 92.7 | 91.5 | 88.4 | 94.7 | Karen et al. [18] (6–10 MHz transducer) |
| 28–35 | 497 | 96 [a] | 83 | 91 | 92 | Nation et al. [4] (7.5 MHz transducer) |
| | | 97 [b] | 86 | 92 | 93 | |
| 29–30 | 138 | 90.4 [c] | 96.0 | 95.0 | 92.3 | Szenci et al. [16] (7.5 MHz transducer) |
| 33–34 | 135 | 96.6 [c] | 98.6 | 98.3 | 97.3 | |
| 33–34 | 135 | 90.0 [d] | 100 | 100 | 92.5 | |
| 30 | 47 | 92.3 | 97.1 | 92.9 | 97.1 | Abdullach et al. [19] |

| Days after AI | N | Sensitivity (%) | Specificity (%) | Positive Predictive Value (%) | Negative Predictive Value (%) | References |
|---|---|---|---|---|---|---|
| | | | | | | (6.5 MHz transducer) |

a Observation of ≥15 mm fluid in the uterine lumen and embryonic membranes; b Observation of an embryo with beating heart; c Recognition of allantoic fluid was used as the criterion for a positive pregnancy diagnosis; d Recognition of an embryo proper with a beating heart was used as the criterion for a positive pregnancy diagnosis.

The reliability of the test greatly depends on the frequency of the transducer used, the skill of the operator [15][20], the criterion used for a positive pregnancy diagnosis (uterine fluid or embryo [3][12][18]; uterine fluid, embryonic membranes, or embryo [4]; amniotic fluid and/or embryo surrounded by an amniotic sac [14]; normal amount of chorioallantoic fluid, embryo, ipsilateral CL [17]; embryo with a heartbeat and corpus luteum [21]; embryo with a heartbeat, fluid-filled uterine horn, ipsilateral corpus luteum [22][23]; amniotic fluid, embryo, or embryonic heartbeat [24]; accumulation of intrauterine gestural fluid in non-pregnant cows [9], and the position of the uterus in the pelvic inlet [25]. More incorrect non-pregnancy diagnoses were made between Days 24 to 38 in cows in which the uterus was located far cranial to the pelvic inlet, in comparison with cows in which the uterus was located within or close to the pelvic inlet [25]. It is important to mention if our ultrasonographic pregnancy diagnoses are based on detection of uterine fluid in the uterus on Day 29 after timed AI, at that time, these cows can be classified 3.8 times more likely as not pregnant 74 days after timed AI than those cows diagnosed pregnant based on visualization of an embryo with a heartbeat [22].

Special attention must be paid to the diagnosis of twin pregnancy by clearly locating the two embryos because twin pregnancy loss and spontaneous twin reduction have been reported to occur up to Day 90 of gestation [26][27]. Possibilities for diagnosing twin pregnancy in the field have been reviewed recently by Szelényi et al. [28].

On the other hand, there is a good correlation between decreasing blood flow during CL regression and progesterone concentration [29][30]. One of the main advantages of CFDU is its high sensitivity (99%) and negative predictive value (98.5%) of diagnosing non-pregnant dairy cattle on Day 20 after AI, which results in few false negative diagnoses [31][32], by monitoring the blood flow in the CL on Day 21 after AI in contrast with measuring the progesterone concentration, non-pregnancy in dairy cows on Day 32 after AI can also be predicted with high accuracy (sensitivity: 99.8%, negative predictive value: 99.3%) [33]. have found that the reduction of blood flow in the CL takes place some days before any detectable changes in CL morphology and echotexture, and therefore, determination of the blood flow and the adjusted blood flow calculated from the ratio of luteal tissue area and blood flow were the best early predictors of non-pregnancy.

Previously three pregnancy proteins (PSPB [34], PAG [35] and PSP60 [36]) have been described, however it turned out lately that the isolated preparations of pregnancy proteins may differ in carbohydrate and sialic acid content, which may explain their minor differences in profile and disappearance from the maternal circulation after calving or EM [18][37][38]. Because the concentrations of these proteins gradually increase during pregnancy, they are good indicators of the presence of a live embryo [39]. Due to the delayed appearance of these proteins in the blood in some cows, their use for PD provides more accurate results from Days 28 to 30 onwards [18][40][41].

A further limitation after late embryonic mortality (LEM) is that protein levels may remain above the threshold level, although the concentration of both proteins decreases steadily [42][43]. This is probably related to the relatively long half-life (7–8 days for bPSPB and 3–4 days for bPAG-1) in the maternal circulation after EM [42][44].

Serrano et al. [45] reported that the herd, fetal sex, milk production, lactation number, and plasma progesterone concentrations did not significantly influence the plasma PAG-1 concentration, while twin pregnancy, the use of Limousin semen and conception during the cool period significantly increased its concentrations throughout gestation. Clone pregnancies comparing with control cows may also significantly increase pregnancy protein concentrations during the whole gestation, regardless of pregnancy outcome [46].

Originally PSPB, PAG-1, and PSP60 radioimmunoassay tests were used to detect pregnancy proteins; however, in the meantime, it turned out that there are 21 PAG family members, and PAG-1 used for pregnancy diagnosis was not the earliest pregnancy protein produced by the trophoblast [47]. After recognizing this, the first in-house sandwich ELISA protocol was developed for measuring the circulating concentration of PAGs by Green et al. [47]. Between Days 26 and 58 after AI, the sensitivity, specificity, positive predictive value, and negative predictive value of the in-house PAGs ELISA tests varied between 94% and 100%, 77% and 94.2%, 90.7% and 97.8%, and 91.2% and 97.1%, respectively (Table 3).

**Table 3.** Accuracy of in-house early-pregnancy associated glycoproteins (PAGs) ELISA tests for diagnosing early pregnancy in dairy cattle.

| Days after AI | N | Type of Sample | Sensitivity (%) | Specificity (%) | Positive Predictive Value (%) | Negative Predictive Value (%) | References |
|---|---|---|---|---|---|---|---|
| 26–58 | 169 | Serum | 97.8 | 91.2 | 97.8 | 91.2 | Piechotta et al. [48] a |
| 27–35 | 2129 | Plasma | 95.1 | 89.0 | 90.1 | 94.5 | Sinedino et al. [49] b |

| Days after AI | N | Type of Sample | Sensitivity (%) | Specificity (%) | Positive Predictive Value (%) | Negative Predictive Value (%) | References |
|---|---|---|---|---|---|---|---|
| 31–35 | 209 | | 98.7 | 88.1 | 83.7 | 99.1 | |
| 26–30 | 106 | | 94 | 77 | NG | NG | |
| 31–35 | 88 | Serum * | 95 | 93 | NG | NG | Friedrich and Holtz [50] |
| 36–40 | 57 | | 95 | 93 | NG | NG | |
| >40 | 128 | | 100 | 92 | NG | NG | |
| 27 | 1673 | Plasma | 95.4 | 94.2 | 90.7 | 97.1 | Silva et al. [17] b |
| 28 | 100 | Plasma | 95.3 | 88.3 | NG | NG | Thompson et al. [51] b |
| 30 | 100 | Plasma | 100 | 90.9 | NG | NG | |

* using a threshold of 2 ng/mL; [a] Used an in-house ELISA assay described by Friedrich and Holtz [50]; [b] Used an in-house ELISA assay described by Green et al. [52] (2005); NG: not given.

Mercadante et al.[53] found higher PAGs concentrations (in-house ELISA) in primiparous cows compared with multiparous pregnant cows (in agreement with Ricci et al. [54] found similar differences between heifers and lactating cows), during the second and later breeding compared with the first breeding postpartum, in cows experiencing clinical metritis, metabolic problems and left displaced abomasum after calving compared with cows not experiencing those clinical diseases and in cases of greater milk yield, while the body condition score did not influence it. [24]. The accuracy of the PAG test (commercial milk ELISA) was not influenced by parity, the number of

days, since the last breeding, and the level of milk production, while Ricci et al. [23] found a negative correlation between plasma and milk PAG concentration (commercial ELISA) and milk production.

There are currently several ELISA tests are available on the market, as discussed below.

BioPRYN ELISA test for the detection of pregnancy-specific protein B in the serum as early as 28 days after breeding in cattle with no interference from a previous pregnancy as early as 73 days after calving.

Between Days 26 to 58 after AI, the sensitivity, specificity, positive predictive value, and negative predictive value of the BioPRYN ELISA test varied between 93.9% and 100%, 87% and 97.1%, 92% and 99.3%, and 91.7% and 97.8%, respectively (Table 4).

**Table 4.** Accuracy of commercially available ByoPRYN pregnancy ELISA tests for diagnosing early pregnancy in dairy cattle.

| Days after AI | N | Type of Sample | Sensitivity (%) | Specificity (%) | Positive Predictive Value (%) | Negative Predictive Value (%) | References |
|---|---|---|---|---|---|---|---|
| 23 | | | 92.8 | 97.0 | | | |
| 23 [a] | 544 | Serum | 98.0 | 97.0 | NG | NG | Martins et al. [55] |
| 28 | | | 100 | 91.6 | | | |
| 24 | 206 | Serum | 100 [b] | 93.6 [b] | 93.3 [b] | 100 [b] | Middleton and Pursley [56] |
| 26–58 | 185 | Serum | 98.0 | 97.1 | 99.3 | 91.7 | Piechotta et al. [48] |
| 28 | 246 | Plasma | 93.9 | 95.5 | 94.7 | 94.7 | Romano and Larson [57] |

| Days after AI | N | Type of Sample | Sensitivity (%) | Specificity (%) | Positive Predictive Value (%) | Negative Predictive Value (%) | References |
|---|---|---|---|---|---|---|---|
| 30 | 229 | | 96.0 | 93.9 | 92.2 | 96.8 | |
| 35 | 246 | | 97.2 | 93.6 | 92.0 | 97.8 | |
| 28–41 | 507 | Serum | 97.0 | 95.9 | 96.2 | 96.7 | Breed et al. [58] |
| 35–60 | 976 | | 96.6 | 92.2 | 95.0 | 94.7 | |
| 30–36 | 1742 | Serum | 95.1 | 68.6 | NG | NG | Gábor et al. [59] |
| 30–36 | 1336 | Serum | 100 | 87.8 | NG | NG | Howard et al. [60] |

[a] A cow was considered pregnant when there was an increase in serum PSPB from basal (Day 17) to Day 23 after AI of more than 28%; [b] Only non-pregnant animals were evaluated.

Martins et al. [55] compared the basal serum PSPB concentrations determined at Day 17 after AI with Day 23 results. They found that if the difference was more than 28% at that time the sensitivity, and the specificity of the ELISA test were higher (98% and 97%), compared to 92.8% and 97% obtained when only Day 23 values were evaluated. It is also important to mention that the concentrations of PSPB on Days 23 and 28 were related to pregnancy losses between Days 28 and 35 after AI, but not to those occurring in later periods (Days 35 to 56 and Days >56 to calving).

Middleton and Pursley [56] suggested comparing the results of serum PSPB samples withdrawn on Days 17 and 24 after AI to diagnose non-pregnant cows with 100% accuracy at Day 24 after AI and to predict early pregnancy loss.

DG29® Bovine Blood Pregnancy ELISA Test for the detection of specific pregnancy-related protein in serum as early as 29 days after breeding in cattle with no interference from a previous pregnancy as early as 90 days after calving. Between Days 28 and 40, after AI the sensitivity, specificity, positive predictive value, and negative predictive value of the D29 test varied between 90.2% and 100%, 66.7% and 98.3%, 91% and 97.4%, and 93.7% and 100%, respectively (Table 5).

**Table 5.** Accuracy of commercially available D29® bovine blood pregnancy ELISA tests for diagnosing early pregnancy in dairy cattle.

| Days after AI | N | Type of Sample | Sensitivity (%) | Specificity (%) | Positive Predictive Value (%) | Negative Predictive Value (%) | References |
|---|---|---|---|---|---|---|---|
| 28 | 100 | serum | 90.2 | 98.3 | 97.4 | 93.7 | Karen et al. [16] |
| 29–36 | 202 | blood | 99.4 | 66.7 * | 92.6 | 96.3 | Paré et al. [61] |
| 30–40 | 212 | plasma | 100 | 81.3 | 91 | 100 | Moussafir et al. [62] |

* 100% in non-inseminated cows.

The IDEXX Bovine Pregnancy ELISA Test for the detection of early pregnancy-associated glycoproteins in the serum or plasma of cattle as early as 28 days after breeding in cows with no interference from a previous pregnancy as early as 60 days after calving. Between Days 25 and ~41, after AI the sensitivity, specificity, positive predictive value, and negative predictive value of the test varied between 92% and 100%, 87% and 100%, 84% and 100%, and 94.2% and 100%, respectively (Table 6).

**Table 6.** Accuracy of commercially available IDEXX bovine pregnancy ELISA tests for diagnosing early pregnancy in dairy cattle.

| Days after AI | N | Type of Sample | Sensitivity (%) | Specificity (%) | Positive Predictive Value (%) | Negative Predictive Value (%) | References |
|---|---|---|---|---|---|---|---|
| 25 | 61 | plasma | 92.0 | 91.6 | 88.4 | 94.2 | Kaya et al. [54] |
| 28 | 84 | | 97.1 | 100 | 100 | 98.0 | |
| 32 | 86 | | 97.4 | 91.4 | 90.4 | 97.7 | |

| Days after AI | N | Type of Sample | Sensitivity (%) | Specificity (%) | Positive Predictive Value (%) | Negative Predictive Value (%) | References |
|---|---|---|---|---|---|---|---|
| 25–32 | 231 | | 95.9 | 94.7 | 93.1 | 96.9 | |
| 28 | 210 | serum | 100 | 94.9 | 94.4 | 100 | Akkose et al. [63] |
| 28 | 320 | plasma | 97 * | 94 ** | 95 ** | 97 * | Mayo et al. [64] |
| 30 ± 1 | 116 | plasma | 100 | 88.9 | 93.8 | 100 | Commun et al. [65] |
| 30 ± 1 | 116 | serum | 100 | 88.6 | 93.8 | 100 | |
| 32 | 141 | plasma | 100 | 87 | 84 | 100 | Ricci et al. [23] |
| 41 ± 2 | 116 | plasma | 100 | 100 | 100 | 100 | Commun et al. [65] |
| 41 ± 2 | 116 | serum | 98.4 | 96.8 | 98.4 | 96.8 | |

\* ±1, \*\* ±2.

The IDEXX Milk Pregnancy ELISA Test for the detection of pregnancy-associated glycoproteins in bovine milk from 28 days after breeding in cows with no interference from a previous pregnancy as early as 60 days after calving. Between Days 28 to ~53, after AI the sensitivity, specificity, positive predictive value, and negative predictive value of the test varied between 96% and 100%, 83% and 97.9%, 79% and 98.5%, and 96% and 100%, respectively

(table 7). While on Day ≥60, the sensitivity and the specificity of the test varied between 98.5–99.2% and 95.5–96.7%, respectively, the positive predictive value and the negative predictive value were 99.8% and 80.8%, respectively (Table 7). It is important to mention that the plasma PAG levels turned out to be approximately twice higher than the milk PAG levels [22].

**Table 7.** Accuracy of commercially available milk pregnancy ELISA tests for diagnosing early pregnancy in dairy cattle.

| Days after AI | N | Sensitivity (%) | Specificity (%) | Positive Predictive Value (%) | Negative Predictive Value (%) | References |
|---|---|---|---|---|---|---|
| 28 | 320 | 96 * | 94 * | 94 * | 96 * | Mayo et al. [64] |
| 28–45 | 497 | 99 | 95 | NG | NG | Dufour et al. [24] |
| 28–35 | 1006 | 99.2 | 93.4 | NG | NG | Fosgate et al. [20] |
| 30 ± 1 | 116 | 98.1 | 90.3 | 94.5 | 96.6 | Commun et al. [64] |
| 32 | 135 | 98 | 83 | 79 | 99 | Ricci et al. [23] |
| 33–52 | 119 | 100 | 97.9 | 98.5 | 100 | Lawson et al. [66] |
| 41 ± 2 | 116 | 100 | 92.3 | 96.4 | 100 | Commun et al. [65] |
| 53 ± 2 | 116 | 98.1 | 96.2 | 98.1 | 96.2 | |
| ≥60 | 683 | 99.2 | 95.5 | 99.8 | 80.8 | LeBlanc [67] |
| ≥60 | 602 | 98.5 | 96.7 | NG | NG | Byrem et al. [68] |

NG: not given, * ±2.

The Rapid Visual Pregnancy ELISA test has been recently developed to detect early pregnancy-associated glycoproteins in bovine whole blood, plasma, or serum as early as 28 days after breeding with no interference from a previous pregnancy as early as 60 days after calving.

Between Days 25 and 45 after AI, the sensitivity, specificity, positive predictive value, and negative predictive value of the visual blood test for dairy cattle were 99.8%, 91.7%, 92.7%, and 99.7%, respectively [69]. When the IDEXX visual ELISA test was used, the sensitivity, specificity, positive predictive value, and negative predictive value of the whole blood test were 98 ± 1%, 85 ± 3%, 87 ± 3%, and 98 ± 1%, respectively [64]. The high sensitivity and negative predictive values mean that very few truly pregnant cows were misdiagnosed as not pregnant. When a microtiter plate reader was used to measure the optical density for individual wells in the ELISA plate, the overall accuracy of the test became 94 ± 1% [64].

The high overall accuracy of the test (98.9%) was reported when the BioPRYN Rapid Visual Pregnancy Test® was used in 92 dairy cattle on Day 28 in heifers and on Day 30 in cows. However, it is important to mention that there were three samples evaluated as 'to be rechecked'; however, later, it was not possible to evaluate them, and therefore, these three samples were removed from the dataset [70]. A somewhat lower overall accuracy (90%) was reported when the Ubio quickVET rapid visual test was used for plasma samples between Days 30 and 40 after AI [62], and a much lower accuracy (70%) was obtained when the Fassisi® BoviPreg visual test kit was used for serum samples on Day 30 after AI [71].

The earliest specific indicator of fertilization and the continuing presence of a viable conceptus is a serum constituent that had originally been detected in mice [72]. This substance is known as the early pregnancy factor (EPF) and has also been described in women [73], sheep [74], cattle [75], and pigs [76].

The reported and extraordinary properties of EPF include:

Early appearance (within hours) after mating or insemination

Rapid disappearance following induced death or removal of the embryos [77][78].

These factors suggest that EPF may be the most useful tool for investigating early embryonic survival or failure [78][79][80]. According to Laleh et al. [81], the rosette inhibition test (RIT) has the potential to distinguish pregnant from non-pregnant dairy cows in the first week of pregnancy. After identifying immunosuppressive EPF with a molecular weight of approximately 200,000 and raising polyclonal antibodies against it [82], a new diagnostic test, the early conceptus factor (ECF) test, was developed in the USA for field use; however, it cannot accurately identify conception within days or at any time before Day 21 of gestation [83][84][85][86][87].

After EPF, a 10.84 kDa protein, chaperonin 10 [88] having immunosuppressive and growth factor properties [89], was identified. Chaperonin 10 belongs to the family of heat shock proteins but, unlike other members of this family, EPF

is detected extracellularly [89]. Further experimental work is needed for the development of an accurate on-farm diagnostic test.

With its very low levels in extrauterine tissues and in the peripheral circulation, IFNT is regarded as a signal for the maternal recognition of bovine pregnancy. INFT contributes to the maintenance of the CL by blocking prostaglandin F2α secretion of the endometrium. Currently, there is no accurate assay for diagnosing early pregnancy based on measuring IFNT concentrations. According to a recent review [2], the relative abundance of ISG in total leukocytes, peripheral blood mononuclear cells and polymorphonuclear cells in pregnant cows from Days 18 to 20 after AI may be significantly higher than in non-pregnant cows.

Another biomarker for diagnosing early pregnancy is the measurement of circulating microRNAs; however, their use is currently limited to research investigations because standardized laboratory techniques are needed to isolate and measure them [2].

Proteomics analysis of the milk identified three possible biomarkers (lactoferrin, lactotransferrin, and alpha1G) for diagnosing early pregnancy [90], while proteomics analysis of the blood identified another three genes (Myxovirus resistance: MX1 and MX2 and oligoadenylate synthetase-1: OAS1), which can be used for early pregnancy diagnosis after validation on a large number of dairy cows [91]. Glycans may also play some critical roles in both the normal function of cells and in disease; therefore, bovine pregnancy can be predicted from a glycan biomarker present in a cow's milk some 2–4 weeks earlier than by the standard method of pregnancy detection using ultrasonography [92]. Circulating nucleic acids (CNAs) or preimplantation factor (PIF) can be another biomarker for diagnosing early pregnancy in dairy cows [93][94], while Barbato et al. [95] suggested measuring PAG-2 mRNA in maternal blood cells, which can be detected earlier than the PAG-1 placental proteins in water buffalo and in other ruminants, as well.

These new branches of diagnostic sciences may contribute to finding molecules that may be exclusively related to maternal metabolic alterations during early embryonic development and to signaling for maternal recognition of pregnancy and continued survival [96], and may contribute to the development of an accurate early pregnancy diagnostic test in dairy cows.

A new technology (in-line milk analysis system, Herd Navigator) has already made possible the automatic collection of milk samples at milking robots or in the milking parlor to analyze progesterone, lactate dehydrogenase, and beta-hydroxybutyrate to detect estrus, tissue damage, and metabolic disorders, respectively [97]. According to Bruinjé and Ambrose [98], by using this new technology for early pregnancy diagnosis, a high sensitivity (>95%) could be reached from Day 27 after AI, while the specificity was somewhat lower before Day 40 after AI. After finding an accurate biomarker for early pregnancy diagnosis, its continuous measurements during milking will make it possible to diagnose pregnancy loss much earlier, meaning we can greatly contribute to increasing reproductive efficiency in our dairy herds. The importance of this technology would also be emphasized by its ability to identify pregnant and non-pregnant animals in a timely manner with no animal handling, because

even a simple transrectal examination of dairy cows can lead to increased plasma and salivary cortisol concentrations and changes in heart rate, heart rate variability, and behavior that are indicative of pain [99].

# 3. Diagnosis of Pregnancy Losses (PL)

One of the advantages of UEs is that PL can be recognized by the absence of a heartbeat, the detachment of the fetal membranes, the appearance of particles in the fetal fluids, or the lack of the embryo proper [9][100]. UEs have revealed that LEM may occur in up to 23% of pregnancies [13][101]. PL (8%) diagnosed by ultrasonography in cows between Days 26 and 58 post-AI occurred at approximately Day 29 (n = 1), Day 33 (n = 3), Day 37 (n = 3), Day 40 (n = 2), Day 44 (n = 1), and Day 56 (n = 1) after AI. The exact day of occurrence of LEM/EFM could not be determined because UE was performed at intervals of 3–4 days [43].

According to Kelly et al. [102], decreased crown-rump length and progesterone concentration measured on Day 34 of gestation tended to be associated with an increased odds ratio for pregnancy loss, whereas CL perfusion and reduced blood flow of the uterine arteries evaluated by Doppler ultrasonography could not be used for predicting pregnancy loss in lactating dairy cattle.

After diagnosing spontaneous cases of LEM by ultrasonography, both plasma bPSPB and bPAG-1 levels began to decline in most cases, while the CL continued to produce progesterone [103][43][44]. This confirms the previous observations [42][104], and demonstrates that lower progesterone concentrations are not the cause of conceptus death.

Although the concentrations of both proteins decrease steadily [42][43] after spontaneous or induced LEM/EFM, they reach the threshold level only after a relatively long half-life, namely, about 7 to 8 days for the bPSPB Thus, they can contribute to the elevation of false positive pregnancy diagnoses on the farm. Although the threshold levels for these tests were not determined, they reached the basal levels in both tests approximately 5 to 7 days after inducing LEM. Based on commercial blood and milk ELISA tests, the threshold levels are reached approximately 7 to 14 days after pregnancy loss [23].

[53] found, that reduced PAG concentrations (in-house ELISA) at Day 32 after AI may predict pregnancy loss between Days 46 and 74 of gestation. Based on positive and negative predictive value analysis, a circulating concentration of PAG (in-house ELISA) below 1.4 ng/mL in lactating dairy cattle following timed AI and 1.85 ng/mL following timed embryo transfer on Day 7 was 95% accurate in predicting LEM/EFM (between Days 31 and 59) at Day 31 of gestation [105]. It has been recently reported that cows being pregnant at Day 31 of gestation and maintaining the pregnancy until Day 59 had significantly higher circulating concentrations of PAG [23]. In contrast, although there was a significant difference in the PAG concentrations measured on Day 24 after AI between pregnant and non-pregnant multiparous cows while in heifers only a tendency was detected at Day 31 after AI, the circulating concentrations of PAG at Day 24 of gestation in animals that maintained pregnancy until Day 60 compared to animals that lost pregnancy between Days 31 and 60 of gestation, were not significantly different [106].

[107] reported that low or very high plasma pregnancy-associated glycoprotein-1 (PAG-RIA) levels on Day 35 of gestation in cows were related to a subsequent pregnancy loss. [108] also found pregnancy losses in cows with high PSPB (commercial ELISA) concentrations ( >1.1ng/mL) and in cows with low concentrations of both PSPB and progesterone (0.6–1.1 and <2ng/mL, respectively), however the prevalence of pregnancy loss was significantly lower in cows with high PSPB concentrations (15%) between Days 29 and 35 of gestation than in those with low concentrations (76.3%).

It has been recently reported that different PAG ELISA assays may accurately detect pregnancy; however, the ability to predict embryo survival vs. mortality during early gestation appears to be antibody-/assay-dependent [109].

In order to be able to decrease the effect of false positive diagnoses, due to pregnancy loss on the farm, it is necessary to repeat the pregnancy tests [110]. By using commercial plasma or milk ELISA tests, the optimal time for the first pregnancy diagnosis is around Day 32 after AI, when plasma and milk PAG levels are at an early peak. After this period, all pregnant cows should be retested on Day 74 after AI or later, when plasma and milk PAG levels rebound from their nadirs [23]. In contrast to the gradual increase in PAG-1 concentration (RIA) throughout gestation [39], plasma and milk PAG levels (commercial ELISA) reached a peak at Day 32 of gestation and then started to decrease to a nadir from Days 53 to 60 for the plasma PAG level and from Days 46 to 67 for the milk PAG level, followed by a gradual increase in PAG levels from Days 74 to 102 after AI [23].

The potential clinical significance of diagnosing pregnancy loss using ultrasonography or pregnancy protein tests and treating the cows with prostaglandin as soon as possible is that these measures may reduce the number of days before re-insemination [111].

# 4. Future Perspectives

Pregnancy diagnosis plays an essential role in decreasing days open in dairy farms; therefore, it is very important to select an accurate method for diagnosing early pregnancy, because the cost of each day open past 100 DIM may reach $4.00 [112] or €2.5 to 6.5 [113], respectively. Besides traditional pregnancy diagnoses made by rectal palpation of the uterus from 40 to 60 days after AI and measuring the serum or milk progesterone concentration between 18 to 24 days after AI [2][114][115], there are several new possibilities to diagnose early pregnancy in dairy farms; however, it is very important to emphasize that before introducing any new diagnostic test we need to make sure the accuracy of that particular test to be able to decrease the rate of iatrogenic pregnancy losses caused by prostaglandin treatment to reduce the interval to the next AI service [114] or resynchronization of the cows [116][117]. Furthermore, the new pregnancy diagnostic results must be confirmed by the old diagnostic method to decrease the negative effects of false negative diagnoses [118]. Linear-array/sector B-mode [119] and Doppler ultrasonography [2] may exceed the other diagnostic methods in the amount of information collecting from each animal during scanning, however, their uses greatly depend on the operator proficiency and availability [119].

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