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REVIEW

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Use and adequacy of non-pregnancy diagnosis in cow. Which future?

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Abstract
In cattle, early detection of gestation is very important from an economic and management point of view in all types of farming. However, due to the poor efficiency of oestrous detection, it is essential to determine non-pregnant cows as early as possible, in order to minimize the inter-insemination interval, thus de facto, reducing herd open days. Direct and indirect gestation diagnostic methods have been developed with the aim of improving the reproductive performance of the herd. Today, the most accurate method for making an early diagnosis of gestation from 28 to 30 days post-insemination is B-mode ultrasound. In recent years, indirect methods have included techniques that allow non-pregnant cows to be identified with a minimum margin of error, the most widely utilized of which is the colour Doppler. This technique is rapidly becoming established for the diagnosis of non-pregnancy, which allows for the identification of non-pregnant animals earlier compared with the pregnancy diagnosis. Some limitations of this technique in dairy cow have been presented.

KEYWORDS
bovine, Doppler, pregnancy, reproductive management, ultrasonography

1 | INTRODUCTION

In the mid-1980s, B-mode ultrasonography began to be used as a complementary examination in bovine reproductive management (DesCôteaux et al., 2009). This technique was initially used for pregnancy diagnosis (Photo 1) (Fissore et al., 1986; Szenci et al., 1999) but soon was utilized to study follicular dynamics, corpus luteum (Acosta & Miyamoto, 2004; Kito et al., 1986), embryonic and foetal development (Curran et al., 1986; Kraisoon et al., 2018), uterine pathologies (Fissore et al., 1986; Meira et al., 2012), gestational losses (Chaffaux et al., 1986; Chaudhary & Purohit, 2012) foetal sexing (Photo 2) (Ali & Fahmy, 2008; Fricke, 2002) physio-pathology of ovary (Photo 3) (Díaz et al., 2019; Gnemmi, 2001; Quintela et al., 2012) and uterus (Bollwein et al., 2002; Debertolis et al., 2016; Sharma et al., 2019) (Photo 4), diagnosis of twin gestation (Photo 5) (Colloton et al., 2010; López-Gatius et al., 2017; Silva del Rio et al., 2009), diagnosis of embryo and foetal death (Photo 6) (Colloton et al., 2010; Gnemmi, 2004b), diagnosis of foetal malformation (Photo 7) (DesCôteaux et al., 2010; Gnemmi, 2004b; Gorjidooz et al., 2021) diagnosis of gestational diseases (Photo 8) (DesCôteaux et al., 2009; Murakami et al., 2019) and recently also to improve the efficiency of selecting donor cows/heifers for embryos and oocytes and to improve efficiency of selecting recipients for embryo transfer (Pugliesi et al., 2018; Stroud & Durocher, 2010).

More recently ultrasonography, thanks to the use of the colour Doppler, is becoming an important tool for diagnosis of non-pregnancy diagnosis (NPD) (Photo 9) (Siqueira et al., 2013), which means to predict the absence of a pregnancy before that the pregnancy diagnosis (PD) itself can be achieved and is based on an indirect assessment of the level of progesterone produced by the corpus luteum by measuring its vascularization surface.

Colour Doppler ultrasonography is accurate to diagnose NPD cows and heifers as early as 20 days after post-insemination (Guimaraes et al., 2015; Siqueira et al., 2013; Viana et al., 2013) or 14 days after embryo transfer (ET) (Palhão et al., 2020; Pugliesi et al., 2014) while pregnancy diagnosis (PD) can be applied at day

28–30 post-insemination (Munoz del Real et al., 2006; Pierson & Ginther, 1984). These differences have an important impact on the income of dairy and beef farming because a 30-day of lost gestation, regardless of the number of lactations and days of lactation, costs about $300 (Cabrera, 2012; De Vries, 2006) and to lose a gestation during the period of 30 to 60 days costs approximately $600. (De Vries, 2006). Thus, the diagnosis of NPD has a fundamental economic significance, because allows the re-insemination of non-pregnant cows earlier compared with the PD (Andrade et al., 2019; Sá Filho et al., 2014). In fact, with NPD is possible to re-inseminate beef cows at day 25 after the previous insemination compared the 32–34 days of the traditional PD (Palhão et al., 2020). In dairy cows, animals diagnosed as non-pregnant at 32 days post-insemination can be re-inseminated at 35 days if they have a corpus luteum and at 42 days post-insemination if they do not have a corpus luteum at the time of PD negative (Sauls & Stevenson, 2017; Wijma et al., 2017).

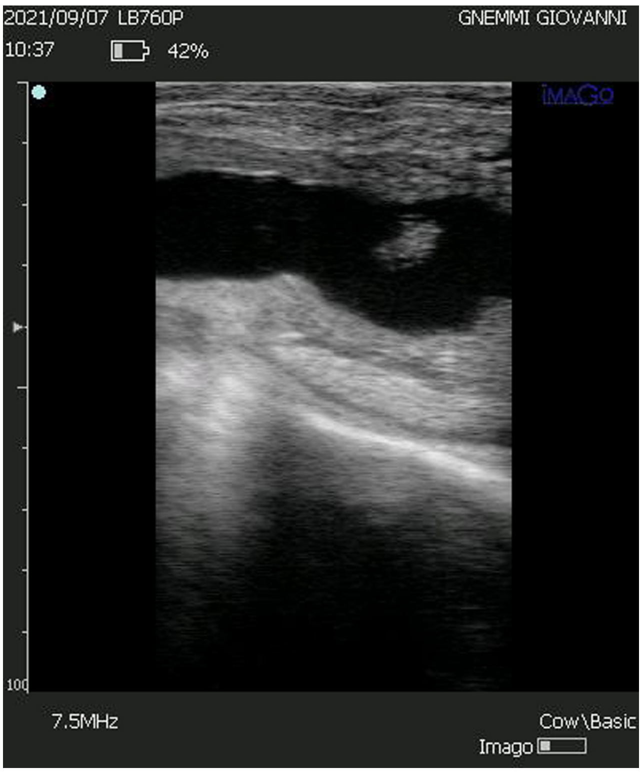

PHOTO 1 Pregnancy diagnosis carried out at 30 days post-insemination in B-mode (echography image by Dr. G. M. Gnemmi)

Therefore, the aim of this article is to describe the NPD by echo Doppler in cows and analyse the advantages and limitations of this technique with particular attention from the point of view of the veterinary gynaecologist working in the field.

1.1 | Determination of pregnancy (PD) and non-pregnancy (NPD) in cow

In cattle, the determination of pregnancy can be done directly or indirectly. Direct methods are based on the identification of the embryo or foetus (Ginther, 1995; Gnemmi, 2004a) and include techniques such as balloting, rectal palpation and B-mode ultrasound diagnosis (Balhara et al., 2013; Christiansen, 2015; Colloton et al., 2010) together with checking of the ovaries for the presence of one or more corpus luteum (CL) and the uterine horns for the presence of fluids. All the above systems can give a good accuracy of pregnancy from day 30 of gestation onwards (Colloton et al., 2010; Fricke et al., 2016; Kastelic et al., 1989). In some reports, has been indicated the possibility to apply transrectal B-mode ultrasound diagnosis at day 25–30 post-insemination with a satisfactory rate of accuracy (Bollwein et al., 2016; Fricke et al., 2002) but not in others (Badtram et al., 1991; Cabrera, 2012; Romano et al., 2006) because there is a quite high rate of false negative (for review see Szenci, 2021). In conclusion, the PD with B-mode ultrasound cannot be applied in field conditions before day 25–30 from insemination. (Gnemmi & Maraboli, 2008).

Other possibility to achieve an earlier pregnancy diagnosis is by the dosage of progesterone in milk and blood (Chebel et al., 2003; Faustini et al., 2007; Martins et al., 2018), by measuring the glycoproteins of gestation (Balhara et al., 2013; Reese et al., 2018; Ricci et al., 2015) or, more recently, by the evaluation of the CL vascularization by the colour Doppler (Siqueira et al., 2013, 2019; Utt et al., 2009). The determination of pregnancy by plasma or milk level of progesterone or glycoprotein plasma levels has a limit that can be still elevated even in non-pregnant cattle, or in animals that have lost gestation from 7 to 14 d after pregnancy loss (Ricci et al., 2015) with the risk of false positive PD. Indeed, when opting for an early diagnosis of pregnancy, it is essential to ensure that the method has a very low false negative rate (Andrade et al., 2019; Pugliesi et al., 2017). The absence of false negative is a key due to the abortion risk when there is the use of hormonal treatments like resynch in cows that are

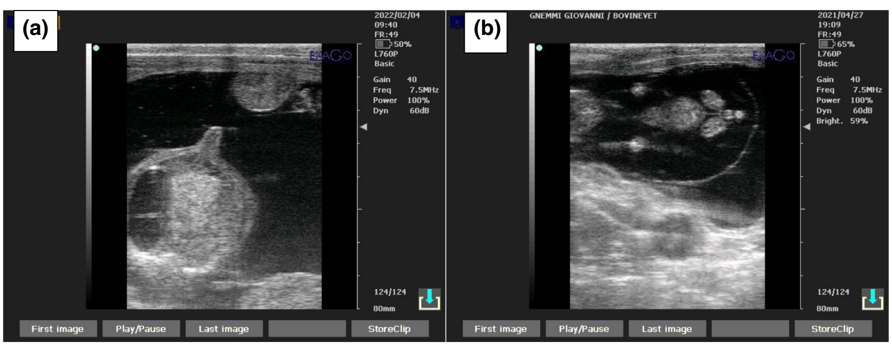

PHOTO 2 Diagnosis of foetal sexing by B-mode ultrasound. (a) Male; (b) female (echography image by Dr. G. M. Gnemmi)

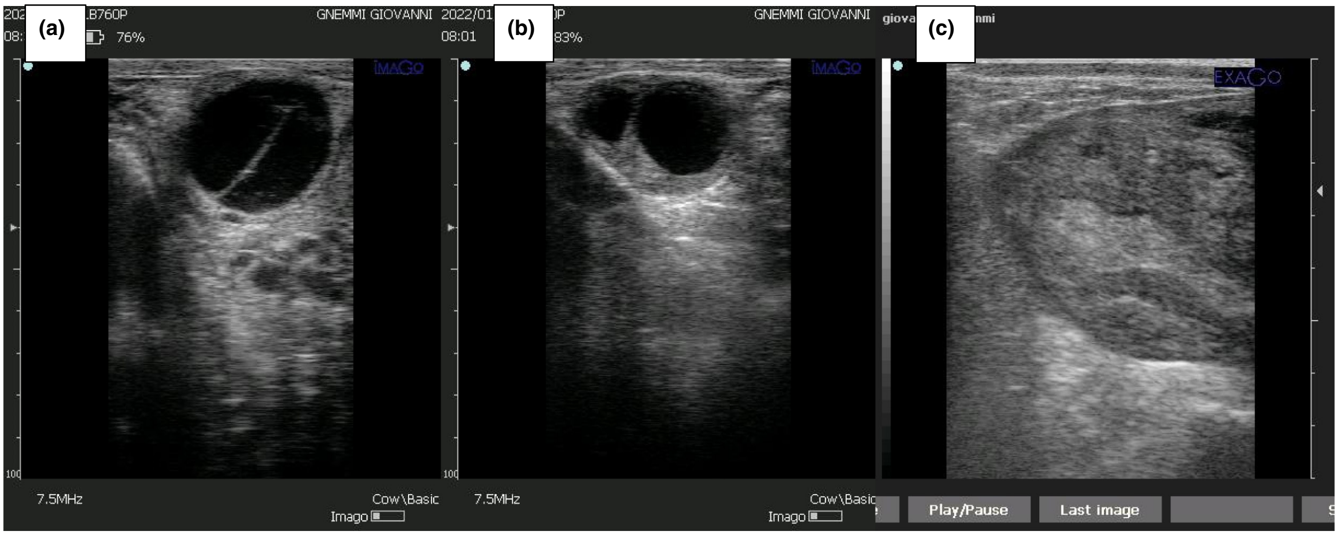

PHOTO 3 B-mode ultrasound diagnosis of ovarian diseases. (a) Follicular cyst; (b) luteal cyst; (c) granulosa cell's tumour (echography image by Dr. G. M. Gnemmi)

pregnant but that are misdiagnosed as being non-pregnant (Pugliesi et al., 2017).

1.1.1 | The colour Doppler application for non-pregnancy diagnosis

Thanks to the colour Doppler, it is possible to evaluate changes of blood flow in CL and therefore the evaluation of functional regression of CL. In this way is possible to predict which animals are not pregnant (NPD diagnosis) by day 18–23 after insemination (Giordano et al., 2012; Siqueira et al., 2013; Utt et al., 2009) or even earlier on day 13 post-insemination (Palhão et al., 2020). In fact, the functional regression of CL anticipates approximately 60–72 hours of the CL anatomical regression (Ginther, 1995; Miyamoto et al., 2005), Thus, at 18–19 days post-insemination, by the evaluation of the functional but not the anatomical regression of CL is possible to predict whether the cow is pregnant or not (Ginther, 2007).

During the meta-estrus, most luteal cells make contact with one or more capillaries, making the CL one of the most vascularized structures in the body (Miyamoto et al., 2005) and at this phase of oestrous cycle, vascular endothelial cells account for up to 50% of the structure of CL (Lei et al., 1991; Miyamoto et al., 2005; O'Shea et al., 1989) and plasma progesterone concentration increases linearly reaching its maximum level on day 14 of the oestral cycle (7.0–8.0 ng/ml) (Herzog et al., 2010).

In a manner mirroring the luteal blood flow increase, plasma progesterone levels increase reaching a maximum level on day 14 and 16 of the oestral cycle (Table 1; Herzog et al., 2010). In the absence of maternal recognition of gestation, cells of the endometrial epithelium produce PGF2α, the main luteo-lysine in the cow (Miyamoto et al., 2005). PGF2α triggers a series of vascular and hormonal processes leading to luteolysis. During the first 3 days of the follicular phase of the cycle (pro-estrus), the luteal blood flow and plasma progesterone (<1.0 ng/ml) concentration are drastically reduced (Herzog et al., 2010).

Despite the different opinions on when to make the NPD using colour Doppler, most of the studies suggest that the use of Doppler for the diagnosis of NPD is most accurate when performed between days 18–21 post-insemination. During this time, in non-pregnant cows, there is a reduction in the CL blood flow that induces a drastic drop in progesterone (Bollwein et al., 2016; Lüttgenau & Bollwein, 2014; Scully et al., 2014).

Thus, echo Doppler is a valuable tool for NPD, but there are some limitations that need to be taken into account:

1. At the timing of luteolysis, there are differences between cows with two follicular waves and cows with three follicular waves. 20% of dairy cows and 80% of beef cows have three waves of follicular growth (Adams et al., 2008). Indeed, the number of follicular waves determines the time at which luteolysis takes place and this determines the length of the oestrous cycle (Adams et al., 2008). Therefore, in two-wave cows, complete luteolysis takes place around day 16 of the cycle, while in three-wave cows, luteolysis takes place around day 19 of the cycle. (Herzog et al., 2010). As consequence, the time of the formation and regression of CL is correlated with the pattern of follicular waves (two or three). When a diagnosis of non-gestation is made by colour Doppler between day 18 and 20 post-insemination, there is the risk of finding, in non-pregnant cows, a CL with a vascularization area of 0.3–0.7 cm2 (≥ 25% peripheral vascularization of the CL) suggesting a pregnancy (Herzog et al., 2010). Therefore, the accuracy of the diagnostic can be reduced due to false positives (Utt et al., 2009).
2. It should not be underestimated that there is an individual difference regarding the vascularization surface of the CL, which further complicates the definition of the best time to perform this

PHOTO 4 B-mode ultrasound diagnosis of mucopurulent endometritis (echography image by Dr. G. M. Gnemmi)

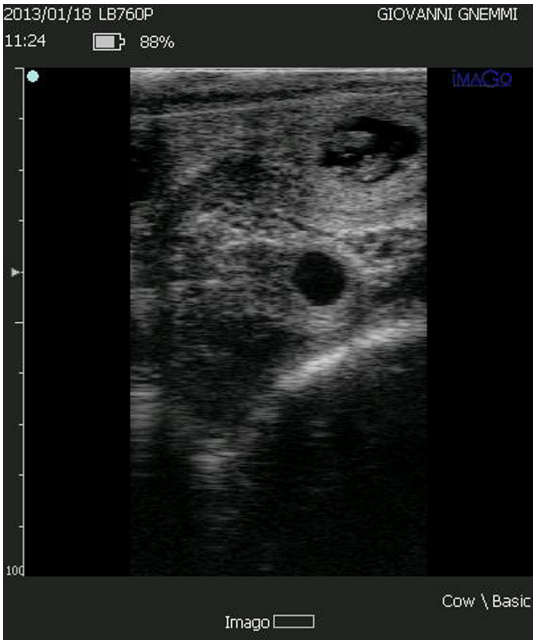

PHOTO 5 B-mode ultrasound diagnosis of twins (echography image by Dr. G. M. Gnemmi)

examination (Herzog et al., 2011; Lüttgenau & Bollwein, 2014; Utt et al., 2009).

3. In high-producing dairy cows, there is a high incidence of the elongated luteal phase (Giordano et al., 2012; Ranasinghe et al., 2011). In these cattle, even in absence of pregnancy, it is normal to find an extensively vascularised CL, even beyond the 20th day post-insemination.
4. In high-producing dairy cows, most embryo losses occur between day 6 and 25 of gestation. When embryo losses occur at day 13–14 post-insemination, luteolysis is delayed, and therefore, it is normal to see a widely vascularized CL in these cows, even beyond day 20 post-insemination (Diskin et al., 2006; McNeill et al., 2006).

PHOTO 6 B-mode ultrasound diagnosis of embryo death (30 days post-insemination) (echography image by Dr. G. M. Gnemmi)

PHOTO 7 B-mode ultrasound diagnosis of foetal malformation (*Schistosomus reflexus*) (echography image by Dr. G. M. Gnemmi)

5. In inseminated cows with subclinical endometritis, or in cows developing endometritis after insemination, a persistent CL may be even in the absence of maternal recognition of gestation (Peter et al., 2009). In these cows, the high levels of PGE2 produced by the endometrial stroma cells block the luteolysis (Mwaanga & Janowski, 2000).

Despite these limitations, the colour Doppler for the diagnosis of NPD between 18 and 21 days is interesting, and the accuracy can be improved by the combination with colour Doppler ultrasound assessment of uterine stratigraphy and by the evaluation of the levels

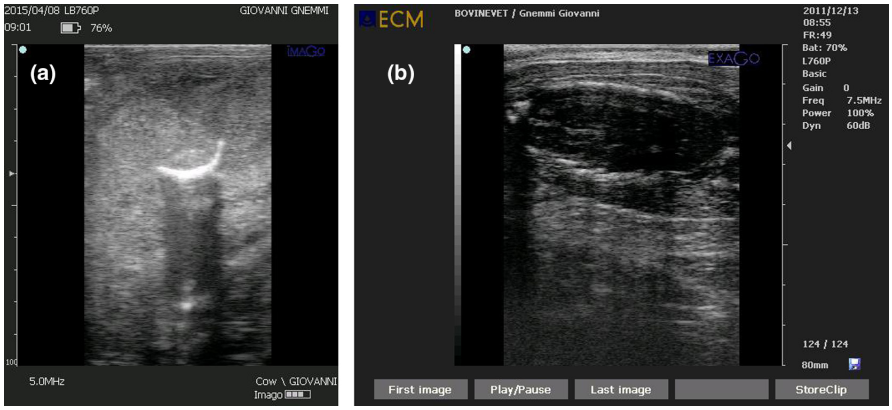

PHOTO 8 B-mode ultrasound diagnosis of pathologies of the gestation. (a) Maceration; (b) mummification (echography image by Dr. G. M. Gnemmi)

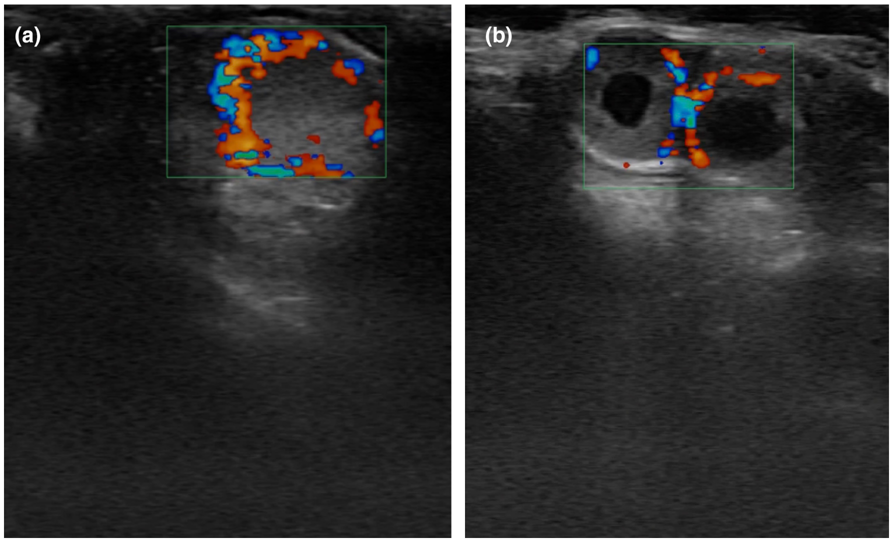

PHOTO 9 Evaluation of LBF. (a) LBF in a cow with progesterone >1.0 ng/ml; (b) LBF in a cow with progesterone <1.0 ng/ml (echography image by Dr. G. M. Gnemmi)

TABLE 1 Progesterone level (ng/ml) and CL vascularization surface area (cm^2), depending on the days of the oestral cycle (processed from Herzog et al., 2010)

Day	P4 (ng/ml)	LBS (cm^2)
4	1.7	0.3
7	4.0	0.7
14	7.3	1.3
16	7.1	1.2
17	5.4	1.0
18	2.7	0.7
19	0.8	0.2
20	0.6	0.1
21	0.5	0.1

of hormones (i.e progesterone), to reduce the margin of uncertainty of NPD.

2 | CONCLUSION

The US has given new insight to determine whether a cow becomes pregnant or not. The B-mode Doppler can allow to individuate the embryo, and this method is the most reliable, but not before day 28–30 of gestation. Conversely, the NPD that is based on the evaluation of CL vascularization by Colour Doppler, allows to identify the cows that are not pregnant before day 28. However, there are some limitations in the use of Colour Doppler for NPD, like the individual difference in the CL vascularization surface, the pattern of follicular waves (two or three), the development of persistent CL or embryo losses.

NPD is important because opened up an excellent opportunity by allowing these cows to be re-inseminated as early as 25 days after the last insemination by the use of resynchronization protocols in this strategy. All inseminated cows receive a resynchronization protocol at around day 22 after AI on day 30. The protocol is cancelled for those diagnosed as pregnant, or completed in non-pregnant ones, and re-insemination occurs within 32 days after the first FTAI (Giordano et al., 2012; Palhão et al., 2020; Sauls & Stevenson, 2017).

Overall, the NPD technique presents some limits and in large commercial dairy herds, it must be carefully taken into account the cost/benefit of this technique before being utilized. Conversely, NPD can be fully justified in small organic dairy farms where is possible for an individual approach to the reproductive management.

The progress of technology may make it possible to create ultrasound units with built-in artificial intelligence to automatically translate the vascular surface of the corpus luteum into ng/ml of progesterone. So, the NPD would then be simpler and of more practical application for the routine reproduction management of the farm.

AUTHOR CONTRIBUTIONS

Our paper is a review and all the authors have been involved in it with a special role for the section of the review in which the author is expert. Furthermore, Gnemmi G decided the topic, proposed the chapters and organized the manuscript; Fabio De Rensis and Roberta Saleri contributed to the overall review of the manuscript; Gnemmi Benedetta and Maraboli contributed to the basic organizations of the manuscript and literature review.

CONFLICT OF INTEREST

There is no conflict of interest, in accordance with the guidance for Authors provided by the publishing house.

DATA AVAILABILITY

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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