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Evaluation of Ultrasonography, Progesterone Analysis, and Relaxin-Based Methods for Pregnancy Detection in Domestic Cats

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Abstract | The present study was designed to compare the efficacy of clinical and laboratory methods in detecting pregnancy at three different time points. Additionally, we aim to determined progesterone (P4) levels during anestrus, estrus, and pregnancy in queens. The experiment was conducted on 30 mature non-pregnant domestic cats housed in a private clinic, Iraq. A total of 25 queens received 100–200 IU of equine chorionic gonadotropin (eCG), while five cats exhibited natural estrus. Estrus determined by monitoring estrous and mating behaviors, as well as changes in vaginal cytology. Blood samples were collected and processed to assess P4 levels during anestrus, estrus, and pregnancy (days 15, 30, and 40). Relaxin hormone levels were evaluated using rapid immune-migration tests during the same pregnancy periods, alongside ultrasonography (ULR). Kittening was observed as the gold standard for comparing pregnancy detection parameters. Diagnostic efficiency was compared using predicted positive and negative values (PPV, PNV), sensitivity (Se), specificity (Sp), and accuracy (Acc). The finding of experiment 1 illustrated that P4 levels were basal during Anestrus but increased significantly ($P \leq 0.05$) within 12 and 24 h after the onset of estrus behavior. On day 15, pregnant cats exhibited higher P4 concentrations than non-pregnant cats, though the difference was not statistically significant. Between days 30 and 50 post-mating, a significant rise ($P \leq 0.05$) in P4 levels was observed, with a clear distinction between pregnant and non-pregnant queens. Non-pregnant queens maintained elevated P4 levels without returning to baseline, indicative of pseudopregnancy. Experiment 2 revealed that P4 assay proved more reliable for pregnancy detection compared to ULR and relaxin testing on day 15. However, by days 30 and 40, all diagnostic values improved, with ULR slightly outperforming P4 and relaxin assays at day 30. Accuracy reached optimal levels for ULR and P4 assays (93%) but was marginally lower for Relaxin. In summary, the P4 levels increased post-mating regardless of pregnancy status, declining after day 30 but remaining elevated above baseline. The P4 assay was reliable throughout pregnancy when using a cutoff point >11.97 ng, however, the low number of non-pregnant queens may have influenced results. In contrast, ULR and relaxin assays were highly reliable from day 30 onward.

Keywords | Ultrasonography, Progesterone assay, Relaxin test, Pregnancy detection, Queen cat

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INTRODUCTION

Queen species are considered a long day breeder, they breed during the long days, and anoestrus takes place during the short days, depend on some factors like attitude and nutrition (Hurni, 1981). For instance, in UK, the peak of reproductive activity recorded in the spring and summer and diminished in the fall and winter (Jennett *et al.*, 2016).

In opposite, according to Faya *et al.* (2011), domestic cats demonstrate estrous cycles all year round in the naturally temperate photoperiod of Argentine.

As the another animal, the hormonal profile during pregnancy is characterized by a sharp decline in plasma estradiol (E2) within the first five days, reaching basal levels, while the plasma P4 values increase similarly in both pregnant and pseudopregnant queens (a condition resembling diestrus without pregnancy) corresponding to the implantation period in the luteal phase (Zschockelt *et al.*, 2014; Johnson, 2022). The P4 increases to over 2 ng/mL beginning 1 to 2 days after ovulation, reach peaks at 21 days of pregnancy, with levels reported after mating ranging from 11-60 ng/mL (Kustritz, 2006) or 15 to 30 ng/mL between 25 and 30 days into the pregnancy according to Feldman and Nelson (2004). In pregnant queens, P4 remains elevated until day 47 of pregnancy, then progressively declines (Mitacek *et al.*, 2015). The primary physiological source is the corpus luteum and placenta, during late pregnancy, the placenta may be the major source of P4 in some animals (Ferre-Dolcet *et al.*, 2018; Schuler *et al.*, 2018).

Diagnosing pregnancy is crucial for determining embryonic and fetal development, predicting the time of kittening (Zambelli and Prati, 2006), assessing fetal viability and losses (Brito *et al.*, 2010), and determining the number of fetuses (Pecchia *et al.*, 2023). Additionally, pseudo pregnancies or non-pregnant luteal cycles can occur in cats following infertile mattings or early embryonic mortality, making the diagnosis of pseudo pregnancy and abnormal pregnancies essential (England, 1998; Bergfelt *et al.*, 2014). Pregnancy can be diagnosed in different species including cats using various methods including clinical techniques such as abdominal palpation, abdominal radiography, and ULR, as well as laboratory methods like analyzing pregnancy-related hormones (Woodland *et al.*, 2014; Younis and Hatif, 2023, 2024).

Several studies demonstrate that while P4 testing is valuable for confirming ovulation in cats, but it has significant limitations for pregnancy diagnosis because P4 levels rise post-ovulation in both pregnant and pseudopregnant queens, the diagnostic Acc when used alone is only 50-60% (Verstegen *et al.*, 1993; Tsutsui *et al.*, 2009).

Abdominal ULR is a non-invasive technique that allows for accurate pregnancy diagnosis at an early stage. An advantage of this method is its ability to assess fetal viability, embryo/fetal number, fetal health, and placental maturity with very high sensitivity and specificity (Mitacek *et al.*, 2015; Muhammad and Aziz, 2021; Younis and Hatif, 2023). However, these diagnostic methods require costly equipment and standard training (Ali and Ghaidan, 2023). The radiation of radiographic techniques could effect on developing fetuses and dams and also have a risk to operators (Orekhova *et al.*, 2024).

Relaxin is a peptide hormone which engaged in several physiological actions particularly during gestation in mammals (Sherwood, 2004). In queen cats, Relaxin concentration is firstly detected at day 14 post mating, it start to rise at the first part of the second trimester reaching a peak on gestational day 32, then declined in the 48-53 days of the pregnancy, it can detect in both urine and serum (de Haas van Dorsser *et al.*, 2006). The detection limit of Relaxin in serum 1.5 ng/mL, and higher than that concentration need for urine samples (de Haas van Dorsser *et al.*, 2007). Therefore, according to same study, high false negative was reported in day 14 and 21 by using urine samples, and the Acc become optimal at day 28 onward.

A study has demonstrated the efficacy of Relaxin testing for early pregnancy detection in cats. The Relaxin test kit was able to identify pregnancy as early as gestational day 20, with a 100% Se from day 29 onward. However, false-positive results were observed in three queens, two of which had large ovarian cysts (approximately 2 × 3 cm), resulting in a test specificity of 95.9% (DiGangi *et al.*, 2010).

Each diagnostic method has a unique feature and the Se, Sp and Acc of each method vary depending on the pregnancy stage, available tools, expertise, and the physiological and pathological conditions of the animals. There is no study has compared hormonal methods (Relaxin and P4 assays) with clinical methods (ULR) for pregnancy detection in queens., This study aimed to compare the P4 levels in anestrus versus estrus and between pregnant Vs and non-pregnant (different periods) in queen cats (Experiment 1). Additionally, the goal was extended to compare the detection values of clinical methods versus laboratory methods for diagnosing pregnancy at three different periods (days 15, 30, and 40 post-mating) (Experiment 1).

MATERIALS AND METHODS

Experimental animals

The experiment was conducted on 30 mature domestic cats from four breeds: Himalayan, Orange, Persian and Shirazi. These queen cats were housed in a private clinic in the Al-Ramadi governorate. The experiment extended from July to October. Six proven breeder males, aged 1 to 2 years, from the Turkish Angora and Persian breeds were reared in a same feeding and room conditions, designated for detecting estrus and mating with the queens.

All queens were double-checked using trans-abdominal

ULR (Chison/China) at two-week intervals to confirm they were not pregnant. A total of 25 queens were received 100-200 IU of eCG (Hingho bsecond Hormone/ Chine) and another five cats were having natural estrus. Estrus determined by monitor the estrus behavior and by changing in the vaginal cytology. When estrus behavior was exhibited by a queen, interaction with a tomcat was permitted after isolated in cat capture boxes under direct observation. After estrus induction and mating, blood samples were collected and processed to evaluate P4 levels using enzyme-linked immunosorbent assay (ELISA). Concurrently, Relaxin hormone levels were assessed using rapid immunomigration tests. and ULR had been conducted to monitor pregnancy at different periods (days 15, 30, and 40 post-mating). Kittening was observed as the gold standard for comparing pregnancy detection parameters.

Gonadotropin treatment

To assess the efficiency of eCG for inducing estrus, all female cats were administrated a single intramuscular injection of 100 IU (20 n) and 200 IU (5 n) eCG. Successful breeding and subsequent pregnancy diagnosis were confirmed using standard veterinary protocols.

Estrus detection and mating

During estrus, the vaginal cytology was utilized to confirm the estrus in cats along with the behavioral. The observed behavioral changes included rubbing their heads and bodies against the ground or inanimate objects, rolling onto their backs, exhibiting lordosis with a raised tail, vocalizing, and calling for males. The vaginal smears were collected two days after the onset of these behavioral changes in cats. The vaginal smear is examined according to Malandain *et al.* (2011) with some modifications. A collection wet cotton swab was kindly inserted and rotated inside vagina. The smear was stained with Giemsa stain (Merck /USA). Additionally, some samples were added to normal saline and centrifuged, the sediment put in slide and examined. Light digital microscope (Olympus/Indonesia) with objective magnifications of 4x, 10x, and 40x was used to observe the stained results. One hundred epithelial cells were examined and grouped according to Termelioğlu *et al.* (2022); it was depending on the prevalence of the cellular type in the oestrus as keratinized superficial cells (nucleated and A nucleated) (>50%) along with low proportion of intermediate cells (Figure 1) Each queen was allowed along with tom cat in a separate place for mating.

Ultrasonography

Ultrasonographic examinations were performed before treatment to ensured that queens were empty and also in days 30 and 40 post-breeding. The examination was done via transabdominal route with micro-convex probe (6 Mhz; resolution: 0.19 mm) to examine the pregnancy (Figures 2-4). The pregnancy was determined by observed the gestational sacs in abdominal cavity, fetal fluid and its structures. The ULR findings demonstrate normal early gestational development in this queen at 15 days post-breeding. A multiple anechoic, rounded structures called gestational sac in different diameter were detected in this stage in just few cases. Findings confirm a viable pregnancy at 30 days' gestation. Fetal and gestational sacs size were consistent with expected developmental milestones. Normal morphology for gestational stage, 6.0MHz transducer. At day 40, fetal bones, ribs, heart, head and trunk appeared clearly.

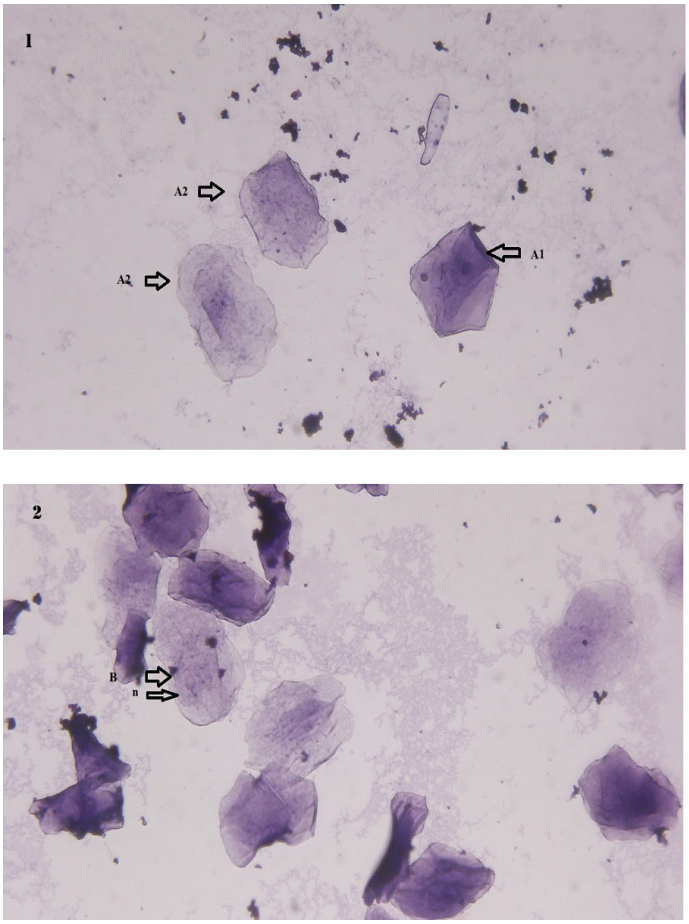

Figure 1: Feline vaginal epithelial cells at estrus showed presence of keratinized superficial epithelial cells with small oval-shaped picnotic nucleus (A1) and without nucleus (A2). 3,4: Feline vaginal intermediate epithelial cell round to oval in shape (B) with smooth cytoplasm and round vesicular nucleus (n).

Blood collection

From each animal, approximately 2-5 mL of blood from the jugular or cephalic vein by a disposable syringe of gauge-20 needle into free-anticoagulant tube was collected before and after breeding. The serum was centrifuged at 3000 rpm for 15 min and the sera were kept frozen into Eppendorf tubes at –20°C until be used for hormonal analysis.

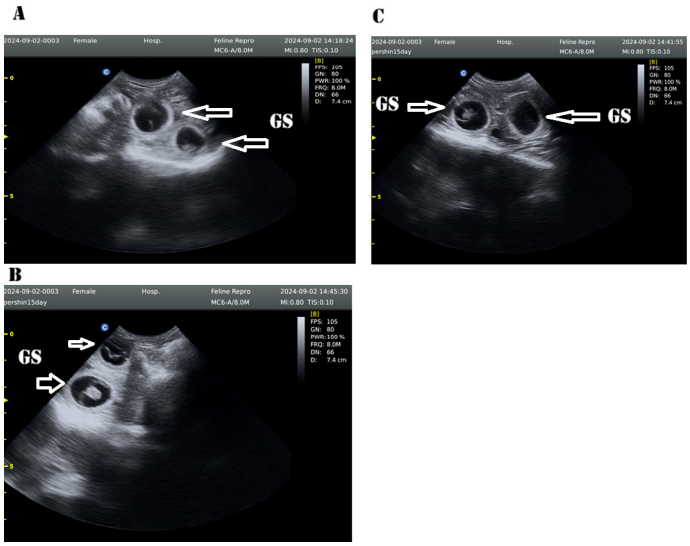

Figure 2: GS: Gestational Sac (Day 15) Anechoic (black) fluid-filled sac seen inside the uterus on day 15 of pregnancy. It appears as a clear black circle with a thin echogenic rim, and is the first ultrasound sign confirming early pregnancy.

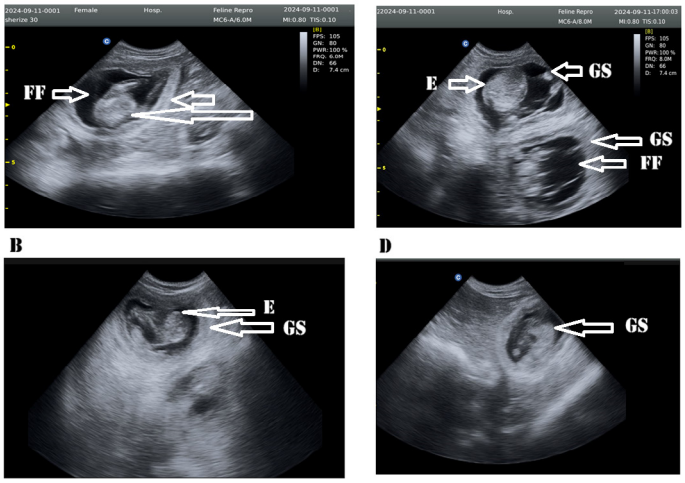

Figure 3: FF: Fetal Fluid, E: Embryo, GS: Gestational Sac (Day 30) This ultrasound image shows the gestational sac (GS) at day 30 of pregnancy. Inside the sac, the fetal fluid (FF) surrounds the developing embryo (E). By day 30, the embryo is more clearly visible, and this stage confirms the pregnancy is progressing normally with identifiable fetal structures.

Hormonal analysis

The serum samples were analyzed to measure the concertation of (Relaxin and P4). Relaxin hormone was quantified using sandwich lateral flow immunochromatography assay (Feline Relaxin Veterinary rapid test /RABID BIOTEC company), whereas the progesterone was evaluated by enzyme link immune sorbent assay kit (Elaere/chine).

Progesterone assay

Blood samples were collected before and after breeding. The serum was separated and preserved at -20°C until the assay. A commercial specific P4 ELISA kit for cats was used to measure P4 levels according to the kit's manual. (Elaere/chine).

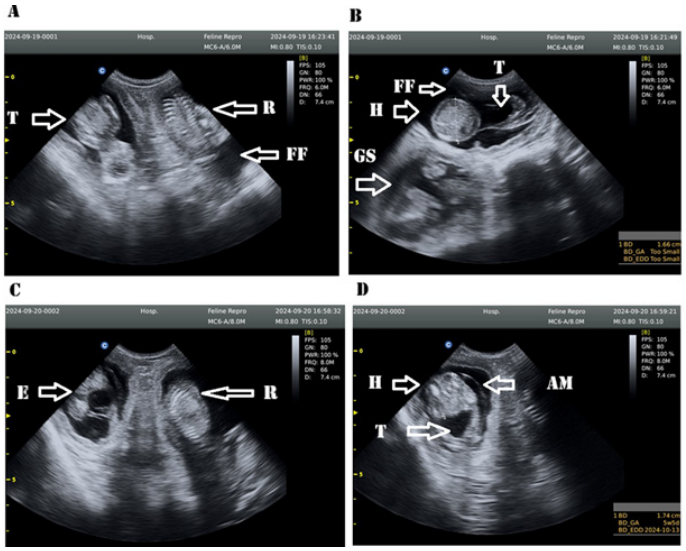

Figure 4: FF: Fetal Fluid, E: Embryo, GS: Gestational Sac, T: Trunk, R: Ribs, H: head, AM: Amniotic Membrane (Day 40). This ultrasound image shows the gestational sac (GS) at day 40 of pregnancy containing the embryo (E) surrounded by fetal fluid (FF). The head (H), trunk (T), and ribs (R) are clearly visible, along with the amniotic membrane (AM). The amniotic membrane is a thin transparent membrane that surrounds the fetus and contains the amniotic fluid, providing protection and allowing normal fetal growth and movement. At this stage, fetal structures are well-developed and easily identifiable on ultrasound.

Relaxin assay

Blood samples were collected after breeding been conducted to monitor pregnancy at different periods (days 15, 30, and 40 post-mating). The Relaxin test was performed immediately using a specific Feline Relaxin Veterinary rapid test (Citest/China) as shown in Figure 5.

Figure 5: Commercially available rapid relaxin test was evaluated for pregnancy diagnosis in 30 cats using serum samples collected at days 15, 30, and 40 of gestation. Results showed the negative (A) and positive results (B).

Statistical analysis

The Statistical Packages of Social Sciences-SPSS (2019) program was used to detect the effect of difference groups in study parameters. Least significant difference-LSD and T-test was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study. ROC curves were used to determine a cut of point for P4 and Se, SP and Acc.

RESULTS

Trial one: Compare between P4 levels during different physiological stages

The Table 1 presented the mean P4 concentrations in female cats during different reproductive stages. During the anestrus phase, when the cat is not in heat and not active hormonally, the P4 level was the lowest, recorded at 0.26 ± 0.05 ng/mL, that reflect the minimal ovarian activity and absence of ovulation. In contrast, during the estrus phase, P4 levels showed a significant rise following mating. At 12 hours post-mating, the concentration increased three times to 0.725 ± 0.17 ng/mL, suggesting the start of hormonal changes linked to ovulation. By 24 hours post-mating, the P4 level reached its highest value at 2.475 ± 0.86 ng/mL, reflecting active luteal function and likely ovulation.

Table 1: Progesterone concentration in Anestrus and Estrus groups of animals in sample study.

Group		Mean ±SD of progesterone conc. (Pg/mL)
Anestrus (5 n)		0.26 ±0.05 c
Estrus	PM: 12 hr. (5 n)	0.725 ±0.17 b
	PM: 24 hr. (5 n)	2.475 ±0.86 a
L.S.D. value		0.417 *

The data in Table 2 compares P4 levels between pregnant and mated non-pregnant cats at different time points. On day 15, pregnant cats showed an average P4 concentration of 11.78 ± 0.65 ng/mL, while non-pregnant cats had 11.37 ± 2.2 ng/mL without a significant difference. However, the P4 concentration in pregnant animals reached 15.47 ± 1.01 ng/mL, which is significantly higher compared to 10.98 ± 1.34 ng/mL observed in non-pregnant animals in day 30 post copulation, the P4 concentration in mated non-pregnant cats still elevated. This can result in a temporary hormonal profile that mimics early pregnancy, a condition often referred to as pseudo pregnancy or false pregnancy. On days 40 and 50 of the reproductive cycle, a significant elevation in P4 levels was observed and the profile continues to show a clear distinction between pregnant and non-pregnant queens. Pregnant queens exhibited a mean P4 level of 16.21 ± 1.71 ng/mL Vs 10.39 ±1.53 ng/mL non pregnant, which is consistent with the hormonal support required for pregnancy maintenance. In day 50; the mean P4 level remained relatively high at 15.04 ± 0.25 ng/mL in pregnant group, while in the non-pregnant group, the level had decreased significantly to 8.72 ± 1.83 ng/mL, also as shown the Receiver operator characteristic (ROC) curves for P4 assay between pregnant and non-pregnant on days 15, 30 and 40 after mating.

Table 2: Progesterone concentration in pregnant and non-pregnant groups of animals with difference periods.

Group	Mean ±SD of progesterone conc. (ng/mL)			
	Day 15	Day 30	Day 40	Day 50
Pregnant (24 n)	11.78 ±0.65 Ab	15.47± 1.01Aa	16.21± 1.71A a	15.04± 0.25A a
Non-pregnant (6 n)	11.37 ±2.2 Aa	10.98 ±1.34Ba	10.39 ±1.53B a	8.72± 1.83B b

L.S.D. value = 1.194 * Means having with the different big letters in same column and small letters in same row differed significantly. * (P≤0.05).

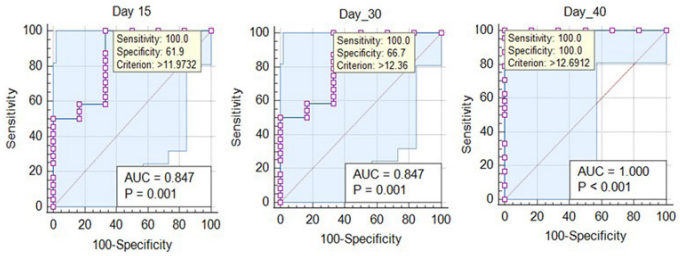

Figure 6: Receiver operator characteristic (ROC) curves for P4 assay between pregnant and non-pregnant on days 15, 30 and 40 after mating.

Compare between the pregnancy detection methods on day 15 post mating in queen cats

Table 3 showed that ULR and Relaxin rapid test were not reliable for detecting pregnancy in cats at 15 days after mating compared to P4 assay. Both ULR and Relaxin showing poor diagnostic performance at this early stage.

Out of 30 queens tested, only two and one were correctly identified as pregnant, while 22 and 23 pregnant cats were misdiagnosed as not pregnant in both ULR and Relaxin rapid test, respectively. This means the Se of ULR was only 8%, indicating that it missed over 90% of actual pregnancies (False negative). However, all six non-pregnant cats were correctly diagnosed, giving the method a Sp of 100%, showing that ULR and Relaxin test are effective in identifying cats that are not pregnant. There were no false positives, meaning any cat diagnosed as pregnant truly was. The overall Acc of the ULR and Relaxin test in this phase was 26% and 23%, respectively, which reflects its poor performance in early pregnancy detection despite perfect Sp.

On the other hand, the overall Acc of the P4 test was 93.9%, showing that it correctly classified the pregnancy

status in nearly all queens. The P4 testing at day 15 using a cut-off point of 11.97 ng/mL is highly accurate and especially reliable in confirming pregnancy, though it may sometimes misclassify non-pregnant queens. The test showed excellent Se, with a value of 100%, meaning that all the pregnant queens were correctly identified and there were no false negatives.

Table 3: Comparative diagnostic efficacy of ULR, P4 and Relaxin for early pregnancy detection (day 15) in queens.

Progesterone (n = 30) Cut of Point >11.97	Relaxin (n = 30)	ULR (n = 30)	Pregnancy status/ Day
80%	100% (1/1)	(2/2) 100%	Predicted + value A/ A+B
20%	20% (6/29)	(6/28) 21.4%	Predicted – value C/ C+D
100%	4% (1/24)	(2/24) 8%	Sensitivity A/A+D
66.67%	100% (6/6)	100% (6/6)	Specificity C/B+C
93.9%	23% (7/30)	26.6% (8/30)	Accuracy

Compare between the pregnancy detection methods on day 30 post mating in queen cats

The results showed that 23 and 22 cats out of 24 were correctly diagnosed as pregnant for ULR and Relaxin test, respectively in day 30 post mating (Table 4). Additionally, six cats were correctly diagnosed as non-pregnant. The PPV was optimum in both methods, indicating that all cats diagnosed as pregnant were truly pregnant. The NPV was 85.7% and 75% for ULR and Relaxin test, respectively, meaning that most cats diagnosed as non-pregnant were actually not pregnant. Sensitivity reached 95.8%, slightly more than Relaxin test (91.6%), reflecting the ability to correctly identify almost all pregnant cases. The Se of both methods was lower than that find in P4 (100%). Additionally, the Sp was 100% in both methods (ULR and Relaxin test), showing perfect Acc in identifying non-pregnant cats. The overall Acc of the diagnosis was 96.6 %, higher than Relaxin test (93.3%). While the Sp of P4 assay was much lower than the rest methods (66.67%), but the Acc still similar in that recorded for Relaxin test. On the other hand, despite the optimal Se, the Sp for P4 assay was lower than above methods (66.67%), that because one third of non-pregnant queen showed high P4 level at this period.

Compare between the pregnancy detection methods on day 40 post mating in queen cats

This study showed that all pregnancy methods were reliable for detecting pregnancy in cats at 40 days after mating. Both ULR and P4 showing excellent diagnostic performance at this period. Among these, 24 cats were actually pregnant. The ULR and P4 showed optimal Se, Sp and Acc. Relaxin hormone test demonstrated highly reliable diagnostic performance with 91% Se, 100% Sp and 93% Acc. However, it was slightly lower than ULR and P4.

Compare between the pregnancy detection methods on day 40 post mating in queen cats

This study showed that all pregnancy methods were reliable for detecting pregnancy in cats at 40 days after mating. Both ULR and P4 showing excellent diagnostic performance at this period. Among these, 24 cats were actually pregnant. The ULR and P4 showed optimal Se, Sp and Acc. Relaxin hormone test demonstrated highly reliable diagnostic performance with 91% Se, 100% Sp and 93% Acc. However, it was slightly lower than ULR and P4.

Table 4: Comparative diagnostic efficacy of ULR, P4 and Relaxin for early pregnancy detection (day 30) in queens.

Progesterone (n = 30) Cut of Point >12.36	Relaxin (n = 30)	ULR (n = 30)	Pregnancy status/Day
80%	100% (22/22)	100% (23/23)	Predicted + value A/A+B
20%	75% (6/8)	85.7% (6/7)	Predicted – value C/C+D
100%	91.6% (22/24)	95.8% (23/24)	Sensitivity A/A+D
66.67%	100% (6/6)	100% (6/6)	Specificity C/B+C
93.3%	93.3% (28/30)	96.6% (29/30)	Accuracy

Table 5: Comparative diagnostic efficacy of ULR, P4 and Relaxin for early pregnancy detection (day 40) in queens.

Progesterone (n = 30) Cut of Point >12.69	Relaxin (n = 30)	ULR (n = 30)	Pregnancy status/Day
100%	100% (22/22)	100% (24/24)	Predicted + value A/A+B
100%	75% (6/8)	100% (6/6)	Predicted – value C/C+D
100%	91.6% (22/24)	100% (24/24)	Sensitivity A/A+D
100%	100% (6/6)	100% (6/6)	Specificity C/B+C
100%	93.3% (28/30)	100% (30/30)	Accuracy

DISCUSSION

The findings of the present study regarding P4 concentrations in cats across different reproductive phases align with previous investigations. The low P4 level observed during anestrus is consistent with earlier reports by Alabodi and Almeeni (2024); they described minimal luteal activity during non-cycling periods in queen. Same data was reported earlier by Ferre-Dolcet *et al.* (2018). Several mating could elicit a sufficient LH release and cause ovulation and increasing P4 along with rapidly declining of estradiol within a 24 hr (Concannon *et al.*, 1989). This hormonal inactivity is a well-established feature of the feline reproductive cycle and has also been supported by Kustritz (2009), who emphasized the absence of significant P4 production in the anestrus stage and increase above 2 ng/mL after several days post copulation from the luteal tissue.

The increase in P4 concentration at day 15 in both pregnant and non-pregnant cats originate from multiple CLs that formed post copulation, these CLs persist even though pregnancy occur or not. Several studies have reported that P4 levels begin to rise shortly after mating in both pregnant and non-pregnant queens, and the P4 levels same in both groups until day 30-40. According to Pineda and Dooley (2003), the P4 level look to be same concentration in both non pregnant and pregnant queens due to persist CLs and decline sharply in non-pregnant from day 21 and becomes basal between days 25 and 40 after mating in pseudo pregnant cats. According to Zschockelt *et al.* (2014), P4 levels in cats begin to increase shortly after ovulation and continue to rise if pregnancy occurs or not, because mRNA expression profiles of steroidogenic enzymes increased during the pregnant and pseudo pregnant cats, the P4 source at this period is CL in both pregnant and pseudo pregnant cats. Another study showed that there was a significant increase in mean luteal cells cell diameters along with pseudo pregnancy progressed (from the 7th day until 25) such as the pregnant queen (Arikan *et al.*, 2009). Additionally, non-pregnant cats that have mated, P4 is also present due to the formation of the CL after ovulation (spontaneous ovulation in 30% of cats). This observation is consistent with the work of Pereira *et al.* (2024), who noted that P4 level is even in the mated pregnant and non-pregnant cats and also in some pathological case such as pyometra.

Regarding to the P4 levels in day 30; In pregnant queens, however, the placenta begins contributing to P4 production, sustaining the pregnancy, therefore, the P4 concentration was higher than non-pregnant. This observation aligns with the findings of Mitacek *et al.* (2015), who noted that P4 concentrations rise steadily from day 21, peaking between days 26 and 28 of gestation (A period coinciding with placental involvement in hormonal support). Similarly, Siemieniuch *et al.* (2012b) have reported that P4 profiles in pregnant and pseudo pregnant cats were comparable during the first trimester but diverged significantly by mid-gestation, with lower levels in pseudo pregnant females. Same authors attribute this decline to the absence of placental P4 biosynthesis in non-pregnant cats.

On days 40 and 50 of the reproductive cycle, a huge difference in P4 levels between the two groups. At this stage, P4 is mainly secreted by the CL, and in some species, the placenta begins to significantly contribute to its production., the P4 level began to decrease gradually in day 50 than day 40. These findings came constant with Mitacek *et al.* (2015) results, which recorded that P4 level still elevated above 14 ng/mL in both periods (days 40-50). Additionally, Ferre-Dolcet *et al.* (2018) reported same results. Siemieniuch *et al.* (2012a) study also support this explanation, suggesting that pseudo pregnancy in queens may last up to 40–45 days post-mating, with hormone levels gradually declining afterward. They showed that serum P4 concentration declining to less than 10 ng/mL with average level 3.8-9 ng/mL was recorded in the gestational days 50th and 56th; however, the P4 values were still around 30 ng/mL in three cats in the gestational days 42 and 56.

Table 3 showed that ULR and Relaxin rapid test were not reliable for detecting pregnancy in cats at 15 days after mating compared to P4 assay. Both ULR and Relaxin showing poor diagnostic performance at this early stage. This finding is consistent with previous studies such as Zambelli *et al.* (2002), which reported that ULR can begin detecting pregnancy reliably from day 18–20 but is most accurate after day 25. Therefore, although this method remains a valuable tool in later pregnancy. The poor body condition, experience of inspector and, efficiency of instrument along with repeated scan may have a role in early detection. Veronesi et al. (2009) reported successful detection of pregnancy as early as day 14, using high-resolution ultrasound equipment. DiGangi *et al.* (2010) and Holst (2022) reported variability in detection success during early gestation and suggested repeating scans for confirmation when performed prior to day 20. Same findings were observed by Zambelli and Prati (2006), who mentioned that high rate of false negatives before day 20. On the other hand, Topie *et al.* (2015) reported successful pregnancy detection in some queens as early as day 14–17 using high-resolution ULR and controlled conditions. However, even in those studies, the authors acknowledged that early detection was not always accurate or practical for general clinical use. Therefore, the results of the present study are largely consistent with the broader consensus

that ULR is unreliable before day 20, despite a few reports of earlier positive detections under ideal circumstances.

The findings from present study on Relaxin hormone testing in cats at day 15 of pregnancy align with previous research. Studies like those by DiGangi *et al.* (2010) and Harris *et al.* (2008) have consistently shown that Relaxin testing can be reliable for confirming pregnancy in animals, but it tends to be more accurate later in the pregnancy. Feldman and Nelson (2004) pointed out that while Relaxin testing is highly specific when it comes to identifying non-pregnant animals, its Se remains low during early pregnancy stages. This is largely because Relaxin levels may not rise significantly enough in the very early stages of pregnancy because it's a placental hormone, which leads to a high number of false negatives, much like the 23 pregnant cats missed in the present study.

On the other hand, DiGangi *et al.* (2010) confirmed that Relaxin testing's Sp is usually very high, meaning that non-pregnant animals are rarely misdiagnosed as pregnant. This aligns with your results, where no non-pregnant cats were falsely identified as pregnant. They also noted that Relaxin tests, when positive, are highly reliable in confirming pregnancy, as that observed in current study with a 100% PPV. However, they acknowledged that such positive results are rare early in pregnancy, as Relaxin levels are often not detectable at this stage.

Furthermore, DiGangi *et al.* (2010) clarified that low NPV of Relaxin testing in early pregnancy because this test unable to detect a pregnancy at such an early stage. This is consistent with the results in present study, where the NPV was low at around 20.7%. The low Se and NPV at this stage of pregnancy are key issues that are echoed in the broader research community.

Based on the studies by DiGangi *et al.* (2010), de Haas van Dorsser *et al.* (2007), and Hussain *et al.* (2024), the present results agreed with the broader scientific understanding of Relaxin hormone testing in cats. All three studies emphasize that Relaxin becomes reliably detectable only after approximately 25 days of gestation and attempting to use Relaxin-based tests earlier such as on day 15 leads to poor Se and a high rate of false negatives. DiGangi *et al.* (2010) study demonstrated that although Relaxin may appear around day 20, consistent detection isn't achieved until day 29. Similarly, de Haas van Dorsser found that urine-based Relaxin detection was ineffective before day 28, which again highlights the importance of timing. Hussain *et al.* (2024) study confirmed that the Acc of Relaxin rapid tests improve significantly after day 25, especially when compared with ULR. More recently, Holst (2022) emphasized the role of Relaxin as the most reliable hormonal marker for pregnancy in cats, particularly when testing occurs between days 20 and 25.

Regarding to efficiency of P4 assay, Siemieniuch *et al.* (2012a) noted that P4 levels during early pregnancy and pseudopregnancy (Persist CLs) often overlap in cats, making diagnosis difficult. Siemieniuch *et al.* (2012b) reported that although P4 increases after ovulation, its variability and the presence of elevated levels in non-pregnant queens limit its diagnostic value before day 20 making it more reliable than P4 in distinguishing pregnant from non-pregnant animals. According to this study, the P4 increases in both pregnant and non-pregnant queens after ovulation and Cl persist regarding pregnant status, but the high selecting cut of point >11.97 of present study and small sample number of non-pregnant may affect on the results.

Comparative diagnostic efficacy of ULR, P4 and Relaxin for early pregnancy detection (day 30) in queens. When compared to previous research, the current findings agreed with Zambelli and Prati (2006) study in regarding pregnancy detection by ULR; which reported that the Se and Sp recorded 93.3% and 90%, respectively when used a high-resolution ULR. Zambelli *et al.* (2002) reported a Se of 91.6% and Sp of 85.7% in early pregnancy diagnosis in cats using ULR, which is slightly lower than the current findings. Additionally, Topie *et al.* (2015) documented an Acc of 94% in mid-pregnancy detection in queens, which, although reliable, is marginally less than the 96.6% Acc observed in this study.

This study demonstrated a high level of diagnostic Acc in detecting pregnancy in cats during the first 30 days of gestation, with a Se of 95.8% and Sp of 100%, indicating a very low chance of false results because the gestational sacs become prominent just in front of the bladder with presence ecogenic structures inside it such as fetal membranes, fetal trunk and its head. These findings are consistent with recent advancements in reproductive diagnostics, which has significantly improved the ability to detect early pregnancies. Similar results were reported by who emphasized the effectiveness of Pecchia *et al.* (2023) modern ULR techniques in feline reproduction, who demonstrated the value of ULR in identifying even complex cases such as monochorionic twin pregnancies.

Regarding to Relaxin test, these results are consistent with earlier studies. For instance first highlighted the reliability of Relaxin as a pregnancy marker in domestic cats, showing a high correlation between circulating Relaxin levels and pregnancy status. More recently, DiGangi *et al.* (2010) confirmed that Relaxin becomes detectable in pregnant queens around day 25–30, emphasizing its use as the first hormone-specific diagnostic tool for feline pregnancy. A study by Andrews *et al.* (2020) reported a

Se of approximately 90% and Sp of 100%, closely aligning with the current data and also slightly lower than the ULR method in Zambelli and Prati (2006).

A comparative study, such as Tsutsui et al. (2009), have emphasized the practicality of Relaxin testing due to its non-invasive nature and high predictive value. However, they also caution that testing too early (before day 25) can lead to false negatives due to undetectable hormone levels in some individuals. A pivotal study by Holst (2022) emphasized that the test could reliably detect pregnancy from day 29 onwards, aligning with previous findings that identified day 29 as the point of maximum test Acc. Tsutsui *et al*. (2009) also mentioned that presence of Relaxin in pregnant queens around day 25–30.

Regarding to P4 assay, the lower Sp (66.67%) indicates limitations, as some non-pregnant cats may still show elevated P4 due to factors like pseudopregnancy or prolonged luteal activity, leading to false positives. Even though, overall Acc (93.3%) is still high, we supposed that the low non pregnant number could affect on the results, it has been lower than the obtained results. On the other hand, the higher threshold (cutoff point) that chosen by statistical program may be rise the Acc. The test's lower Sp means it should ideally be combined with other diagnostics, such as Relaxin assays or ULR, for definitive pregnancy assessment.

The observed P4 performance (100% Se but 66.67% Sp at >12.36 ng/mL) reflects well-documented physiological and diagnostic challenges in feline reproduction. The perfect Se aligns with Verhage *et al*. (1976) study, which demonstrating that P4 level for pseudpregnant cat began to decline significantly than pregnant cat, that improve the Acc of detecting non pregnant in cats. Zschockelt *et al*. (2014) showed decreased in P4 level with progression of the pseudopregnant luteal phase compared to pregnant.

England (2010) demonstrating that feline corpora lutea maintain P4 >10 ng/mL throughout gestation, making subthreshold levels reliably exclude pregnancy. However, Tsutsui's *et al*. (2009) showed that 20% of non-pregnant queen's exhibit prolonged luteal activity P4 levels indistinguishable from pregnant animals. This biological phenomenon explains the 33.3% false-positive rate in present Table 4. Ultrasound examination in cats at Day 40 of pregnancy indicates a very high reliability in confirming pregnancy because it can visualize the anatomical structures such as skeletal formation, organ development, and fetal movement. These results are consistent with the findings of Zambelli and Prati (2006), who reported clear visibility of fetal structures, including kidneys and the cerebral choroid plexus, by day 40 of gestation in queens. Additionally, Topie *et al*. (2015) confirmed that high-frequency ULR probes enhance the visualization of fetal details, heartbeat and fetal movement supporting the precise identification achieved in this study. They reported Se and Sp reaching 100% depending on the operator's experience and equipment quality. Furthermore, research Mitacek *et al*. (2015) supports this as well, stating that by Day 40, ULR provides clear fetal images with reliable cardiac activity detection.

At Day 40 of pregnancy, the Relaxin hormone test demonstrated highly reliable diagnostic performance and confirming its strong reliability in differentiating between pregnant and non-pregnant states in queens at this gestational stage. These findings emphasize the value of Relaxin hormone testing as a dependable method for pregnancy diagnosis in cats during mid-gestation. de Haas Van Dorsser *et al*. (2007) confirmed these findings, noting that Relaxin is produced by the placenta and serves as the only pregnancy-specific hormone in cats, with high diagnostic value from day 25 onward. More recently, Bergfelt *et al*. (2014) found that commercial Relaxin tests demonstrated near-perfect diagnostic performance when used after day 30 of gestation, especially around day 40, where hormonal levels plateau and provide stable readings.

Along with ULR, the results for pregnancy detection by P4 in queens at day 40 show excellent diagnostic performance. Progesterone levels using a cutoff of >12.69 ng/mL demonstrated optimal Se, Sp and Acc. While, Relaxin test showed slightly lower performance, but it maintains as a good confirmatory test but with a small chance of false negatives. These findings suggest that day 40 represents an ideal window for pregnancy diagnosis in queens, where multiple diagnostic modalities reach their peak reliability. The data also implies that by this stage, non-pregnant queens have undergone complete luteal regression, eliminating the false positives that can occur with P4 testing in earlier stages of the reproductive cycle.

The exceptional performance of P4 testing at day 40 (100% Acc across all parameters) builds on the work of Mitacek *et al*. (2015) who established that mid-gestation P4 levels in queens show remarkable diagnostic stability. The complete absence of false negatives in our data corroborates Siemieniuch *et al*. (2012b) findings about P4 maintenance in viable feline pregnancies beyond day 35. The perfect Sp resolves earlier diagnostic challenges noted by Verhage *et al*. (1976), where luteal persistence caused false positives in earlier gestation stages.

CONCLUSIONS

The P4 level increased gradually after estrus and after mating in all pregnant and non-pregnant queens. The presence of elevated P4 in mated non-pregnant cats is a normal

physiological response due to ovulation and CL formation. The effectiveness of pregnancy diagnosis in queens varies by method and gestational stage. Progesterone testing can aid early detection but with very high cut of point to avoid the false negative results. Ultrasonography and Relaxin serve as pregnancy-specific markers, with reliable detection beginning from day 30 post-mating. Using a combination of these methods enhances diagnostic confidence and improves reproductive decision-making in feline practice.

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NOVELTY STATEMENT

Specific P4 analysis, specific Relaxin testing, and abdominal ULR) across key gestational timepoints; days 15, 30, 40, and 50 in domestic queens

AUTHOR'S CONTRIBUTION

MIM: Study design, practical work, ultrasound examination, data collection, and writing of the manuscript. LSY: Statistical analysis, laboratory support, review, and editing.

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Data availability

The data supporting the findings of this study are available upon request from the corresponding author.

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The author(s) declare that no Generative AI was used in the creation of this manuscript

Conflict of interest

The authors have declared no conflict of interest.

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