DETECTION OF SERUM RELAXIN AS A DIAGNOSTIC TOOL FOR EARLY PREGNANCY DIAGNOSIS IN BITCHES

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Thesis submitted in partial fulfilment of the requirement for the degree of

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DECLARATION

I hereby declare that this thesis entitled **DETECTION OF SERUM RELAXIN AS A DIAGNOSTIC TOOL FOR EARLY PREGNANCY DIAGNOSIS IN BITCHES** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree diploma associateship fellowship or other similar title of any other University or Society

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CERTIFICATE

Certified that the thesis entitled **DETECTION OF SERUM RELAXIN AS A DIAGNOSTIC TOOL FOR EARLY PREGNANCY DIAGNOSIS IN BITCHES** is a record of research work done independently by **Dr Deepthi L** under my guidance and supervision and that it has not previously formed the basis for the award of any degree fellowship or associateship to her

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DEEPTHI L

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We Love Dogs and Respect their Nature,

To us, a dog is far more than "Just a Pet"

It is a member of our family!

But it is not a four legged human

And it is not a Toy.....

Introduction

1 INTRODUCTION

Dog has been the best friend and companion of man at all t mes It has the unique distinction of being the first animal to be domesticated owing to its value as hunting partner In the early days of domestication dog had no economic usage to man but gradually it diffused into human social linkages and became a utility animal as an object of sacrifice on special occasions employed as a guardian at home and fields to fold sheep and cattle as a hunting partner bes des being used as detective agent for solv ng criminal offences

Today dogs are reared as pets of high esteem fascinating and delightful companions rather than to do any specified job at the call of the r masters There are many stor es about dogs in all countries of the globe with regard to its love affection and fidelity to human beings The social bond between man and dog is very strong and an everlasting one Love of a dog is thrust upon man voluntarily or involuntarily at all stages at all times

India is undergoing rapid socio economic changes due to globalization and one of the changes evident in the animal husbandry sector in urban areas is the increase in the purebred canine population as dog is considered as the best companion in solitude and periods of trouble More than 40 exotic dog breeds are at present available in Kerala Since prof tability of dog keeping mainly depends on optimum reproductive efficiency pregnancy diagnosis holds nuch importance

The time has come and most of the dog owners have realized the importance of dog breeding and its economic benefits Today dog breeding is a lucrative venture It has prov ded a lot of opportunities especially for youngsters and pet lovers to take up dog breeding systematically on a large scale through establishment of kennels maintenance of highly selective stud dogs and b tches in the mode of a private sector And in this context the role of veterinar ans holds

importance especially the veterinarian s involvement related to breeding management

Now a days the role of veterinarian in managing dog breeding s growing in demand Breeding management is an important component of successful planned reproduction in dogs We see more variability n normal canine reproductive cycles than in any other domestic species This normal variability sets up inevitable problems when practitioners and breeders try to manage breed ng A thorough knowledge regarding the breeding and infertility in dogs will help a veterinarian to apply available knowledge and technology scientifically for better reproductive management Breeding management n dogs has undergone dramatic changes in the last 10 to 15 years Because of the great variability among bitches and their cycles flexibility is the key word to canine breeding management

An early and reliable pregnancy diagnosis not only reduces the chances of accidental mishap but also offers an opportunity for better management and nutrition for the pregnant bitches The complete evaluation of canine pregnancy entails confirmation of pregnancy estimation of litter s ze and assessment of foetal viability The current expectation of breeding management by a veterinarian include supplemental services such as early and accurate pregnancy detection estimation of stage of gestation est mation of litter size attending any complications encountered at the time of whelping and last but not the least the post natal care All these require a working knowledge of b ological and physiological progress of normal canine pregnancy and also about the diagnost c and therapeutic modalities applicable to pregnant bitches

More variability in the normal canine reproductive cycle can be observed than in any other species The bitch is a monoestrous non seasonal polytocous animal that ovulates spontaneous at the end of a variable follicular phase lasting from 4 to 28 days (England 2001) Reproductive activity in the bitch differs from the polycyclic pattern of other spec es in that there are no frequent recurring

periods of heat During each cycle the bitch has prolonged follicular and luteal phases compared to those of the other cycling species of farm animal

The inter oestrous interval is identical in pregnant and non pregnant cycle therefore pregnancy cannot be anticipated by a failure of return to oestrus The main problem in diagnosing pregnancy in bitches is that overt pseudo pregnancy is very common which exhibit the behavioural and physical changes simulating pregnancy Many techniques are available to know whether a bitch is pregnant or not but documented evidence of comparative studies on different techniques of early pregnancy diagnosis are meagre Looking for behavioural as well as physiological changes abdominal palpations are some of the earliest routine methods (Arthur *et al* 1996)

Acute phase protein estimation ultrasound scanning (Kutzler *et al* 2003) and endocrine assay are some of the techniques adopted now a days Few authors have reported that serum relaxin concentration increases dur ng pregnancy and is a specific ndicator of pregnancy in bitch

The present study entitled Detection of serum relaxin as a diagnostic tool for early pregnancy diagnosis in bitches was undertaken to detect the serum relaxm compared with abdominal palpation and ultra sonography for evolving a suitable easiest and reliable method for early pregnancy diagnosis in bitches

Review of Literature

2 REVIEW OF LITERATURE

Unusual breeding and variations in the oestrous cycle from the average duration are the most common causes of infertility in bitches Hence t is recommended that all the bred females should be examined for pregnancy diagnosis as early as possible The unusual reproductive physiology of the b tch is characterized by poor correlation between the behavioural s gns of oestrus and hormonal changes increased plasma progesterone concentrat on prior to ovulation gestational length varying between 58 to 72 days and usual occurrence of pseudo pregnancy (England 2001)

2 1 THE NORMAL REPRODUCTIVE CYCLE OF THE BITCH

The normal reproductive cycle of the bitch is comprised of four stages proestrus estrus diestrus and anoestrus (Concannon *et al* 1986)

2 2 FINDING OPTIMAL TIME FOR BREEDING IN BITCH

There are several methods for identifying optimal breeding time in b tch

There is considerable variat on in the time of ovulation in relation to the onset of vulval swelling and sero sanguineous discharge of early proestrus in bitches Therefore breeding at pre determ ned days after onset of proestrus n bitch would result in apparent infert lity (Concannon *et al* 1983) But England (1989) opined that the period of peak fertility for natural mating ranged from one day before and until six days after the LH surge

England (2001) reported that for assessing the optimal breeding t me for mating in bitches was by observing the timing of vulval softening which often occurs at the time of LH surge when there was a change from high to low estrogen concentration Further it is suggested that serial monitoring of plasma progesterone concentration allowed anticipation of ovulation confirmat on of ovulation and detection of fertilization period

England and Concannon (2002) opined that onset of behavioural estrus timing of vulval softening and exfoliative vaginal cytology were the cheapest and simple methods used to determine the optimal time for breeding But Sreedevi (2005) had suggested that vaginoscopy was one of the best tools for determin ng the ovulation in bitches

2 2 1 Clinico Gynaecological Examination

Concannon *et al* (1983) found that some bitches might exhibit phantom proestrus by displaying little or no outward signs of bloody discharge making it difficult to estimate the average date of ovulation

England (1998) observed that distinct vulval softening occurred at the time of LH surge and suggested that mating should be commenced four days after the onset of vulval softening However Hewitt and England (2000) opined that behaviour of the bitch in response to the dog and the softening of vulva could help to estimate the time of ovulation since both of these events occurred approximately two days before ovulation

Purswell and Parker (2000) found that vaginoscopy was the most accurate diagnostic breeding tool when compared to vaginal cytology However Romagnoli (2006) op ned that performing vaginal cytology and checking the bitches behaviour for the onset of male receptivity were the two most pract cal ways for determining the best time for breeding

2 2 2 Exfoliative Vaginal Cytology in Bitch

Exfoliative vaginal cytology (EVC) n the bitch provides a useful marker about the degree of vaginal mucosal proliferation that in turn is influenced by the changes in the plasma estrogen and progesterone concentration Therefore EVC is a fairly simple method of monitoring the stage of oestrous cycle in this species

Olson *et al* (1987) reported that 90 percent or more of the epithelial cells in a smear from an oestrus bitch would be superficial cells The authors also opined that the smears obtained during that period would not contain neutrophils Erythrocytes diminished in number but could be observed through out estrous and also during early diestrous in many bitches

Wright (1991) evolved the relationship between the time of ovulation and the day of the cycle by calculating the vaginal cell eosinophilic index (EI) reaching 100 percent Feldman and Nelson (1996) opined that breeding should be attempted throughout the period when more than 80 percent of epithelial cells are cornified But Simon and Athman (1998) found that an abrupt clearing of background ndicated the occurrence of LH surge Becha (2000) observed that during proestrus the percentage of superficial cells increased until it was nearly 100per cent by the beginning of oestrus

Hewitt and England (2000) mentioned that the optimal time for breeding was when the cornification index is 80 percent or more and also found that fertile period could be predicted by calculating the percentage of epithelial cells that appear anuclear when using a modified Wright Giemsa stain According to them

Anuclear cell index No of anuclear cells / Total No of epithelial X 100 cells

Purswell and Parker (2000) found that vaginal cytology finding had a characteristic feature during oestrous The superficial cell type predominated wh te blood cells present were low in number and the RBC present was few in number But according to Arthur *et al* (2001) the fertile period could be predicted by calculating the percentage of epithelial cells that appeared cornified using a modified Wright Giemsa stain England (2001) also suggested that the fertile period could be predicted by calculating the percentage of epithelial cells that appeared anuclear when using a modified Wright Giemsa stain Mating should be allowed throughout the period when more than 80 percent epithel al cells were anuclear

Johnston *et al* (2001) found that greater than 90 percent of the epithelial cells were of superficial type at the time of maximum cornification The authors also opined that the absence of neutrophils and the presence of numerous bacteria during estrus should be considered normal But according to England and Concannon (2002) the fertile period could be predicted by recording the percentage of epithelial cells that were superficial when stained using a Wright Giemsa stain and the percentage of cornified cells might surpass 80 90 percent and reached nearly 100 percent

2 3 METHODS OF PREGNANCY DIAGNOSIS IN BITCH

A number of techniques can be used to diagnose pregnancy in bitch It is apparent that there is a difference between the actual and apparent length of pregnancy

2 3 1 Behavioural Changes

Jones and Joshua (1982) found that many pregnant b tches showed no change in temperament and behaviour and remained active unt l and unless gross abdominal enlargement developed However England (1998) recorded that both pregnant and non pregnant bitches exhibited behavioural changes typical of pregnancy But Thou (1999) opined that although no behavioural changes were observed until 30 35 days of gestation few bitches showed signs of nappetence

England (2001) stated that pregnant and non pregnant bitches might exhibit behavioural changes typical of pregnancy Food intake usually increased by approximately 50 per cent in second half of pregnancy however it was not uncommon for pregnant bitches to have a brief period of reduced appetite commonly encountered about 3 4 weeks after mating

2 3 2 Body Weight

Schroeder and Smith (1995) conducted a study on body weight and feed intake of German shepherd bitches during pregnancy and lactation and noted that

from the 28th day of pregnancy the bitch maintained a slow and steady increase n body weight which was found to be 0 0849 kg/day England (1998) also op ned that in pregnant animals the total body we ght increased to 20 55per cent dur ng gestation

According to Thou (1999) there was a steady increase n the body we ght as the pregnancy advanced ranging from 21 36 kg in pregnant Alsatian as aga nst 19 30 5 kg in non pregnant and 9 12 kg in pregnant Pomeran an as aga nst 8 10 5 kg in non pregnant

Arthur *et al* (2001) stated that grav d uterus and ts contents caused no appreciable increase n body weight during the first five weeks of gestat on After f ve weeks body weight rapidly increased according to the number of the fetuses The increase in body weight varied from 1kg in a 5 kg bitch to 7 kg or more n bitch weighing 27 kg But England (2001) opined that body weight began to increase from day 35 onwards and increased up to 50 percent of normal weight and observed that these changes might not be obvious n bitch with small l tters

2 3 3 Pregnancy Diagnosis by Transabdominal Palpation

Harrop (1960) described in detail about the pregnancy d agnos s by palpation at different stages of gestation and found that the opt mum per od for the early diagnosis of pregnancy in the bitch was 24 to 30 days The embryos were noted to be spherical in shape and about one inch in d ameter to the touch

Sokolowski (1980) stated that palpat on of the abdomen between day 20 and 28 after breed ng was helpful to diagnose pregnancy and noticed that by day 20 the develop ng uterus had spher cal swellings approx mately 10 to 15mm n diameter After day 28 it was extremely d ff cult to palpate the pregnant uterus because the spherical shape of the uterine enlargements changed to an ovo d shape with an increase in size to 15 to 30 mm in d ameter depend ng on the s ze of the bitch

According to Shille and Gontarek (1985) the early pregnancy detection in the bitch was generally limited to transabdominal palpation of the gravid uterus between days 25 to 36 after breeding since the gestational vesicles were poorly distinguished from other viscera fecal material or diseased uterus before and after these dates Tavern (1985) also found that the non pregnant uterus and the pregnant uterus before day 21 of diestrous were not reliably palpated in most dogs

Gangadhar (1995) found that the optimum time for pregnancy diagnos s by abdominal palpation was 28 days after breeding and recorded the size of each foetal swelling as two cm in diameter But Arthur *et al* (1996) observed that on days 18 to 21 the embryos represented a series of tense oval distensions in the cornua about twelve mm long and nine mm broad and found it difficult to detect embryos in large and fat bitches at this stage by palpation Feldman and Nelson (1996) also found that palpation of abdomen was easy inexpensive and reliable in recognizing pregnancy between days 20 and 30 of gestation and uter ne swellings at individual placental sites were usually palpable at this stage

England (1998) stated that the optimum time to do abdominal palpation in bitches was one month after mating and at this stage the conceptuses were spherical in outline and varied between 15 and 30 mm in diameter and proved t difficult to do abdominal palpation after day 45 of gestation

Thou (1999) recorded earl est results of pregnancy d agnosis in bitches by transabdominal palpation on 21 day after breeding in a Pomeranian bitch By about 30 to 35 days post breeding the accuracy of palpation in Alsatian and Pomeranian was 88 9 and 100 percent respect vely But Gradil *et al* (2000) palpated pregnancy at 26 to 28 days post breeding and recorded that by day 28 uterine swellings were of three to five cm in diameter for middle sized bitch After day 30 of gestation the uterus enlarged rapidly and occupied a more cranioventral position and it became more difficult to palpate as discrete swellings

Purswell *et al* (2000) also reported that abdominal palpation was best accomplished by 25 to 28 days when the strings of pearls effect of the uterus was most obvious After 30 to 35 days post breeding the uterine swellings were elongated become more oval and more fluctuant At this time it was difficult to differentiate a gravid uterus from other abdominal contents

Johnston *et al* (2001) opined that the bitch should be palpated approximately 31 33days after the LH surge or about 28 30 days after suspected day of ovulation The embryos and the chorio allantoic vesicles in the bitch formed a series of ovoid swellings in the early gravid uterus the most caudal of which could be identified by palpation through the abdominal wall as early as 17 22 days after ovulation Arunmozhi (2005) also recorded the earliest correct diagnosis of pregnancy by abdominal palpation by 22 days after first mating But Asha (2005) found that pregnancy diagnosis by transabdominal palpation at 20 30 days post breeding was difficult as the distinct uterine swellings could not be appreciated from the abdominal viscera in bitches

2 3 4 Accuracy of Pregnancy Diagnosis by Abdominal Palpation

According to Allen and Meredith (1981) palpation of the abdomen in the period from 26 to 35 days after mating was found to be 87 percent accurate in diagnosis of pregnancy Earliest pregnancy detection was obtained at 21 days after first mating but correct positive results were obtained in only 52 percent from day 21 to day 25 post breeding by palpation During period from day 25 to 35 the accuracy was 75 per cent

Out of 55 b tches examined for pregnancy by Toal *et al* (1986) the accuracy of pregnancy detection and foetal counting by abdominal palpation was 88 percent and 12 percent respectively and stated that palpation was less rel able than ultrasound for determining pregnancy and litter size

According to Allen *et al* (1991) abdominal palpation was more accurate from 24 to 35 days post breeding Deka *et al* (2004) also reported that earliest pregnancy could be diagnosed by abdominal palpation on 23rd day post service with 65 to 71 percent accuracy which improved to 100 percent from 28th day to term

In the study conducted by Asha (2005) among large medium and small breeds the accuracy obtained by abdominal palpation was 61 9 60 and 75 percent respectively But when the palpation was done in between 31 to 40 days post breeding the accuracy was 66 6 100 and 80 percent respectively

2 3 5 Pregnancy Diagnosis by Ultrasound Scanning

Bondestam *et al* (1983) reported difficulty in detecting fetuses by 21 days of gestation and suggested ultrasound scanning from 28 35 days of gestation The authors recorded that all fetuses detected before 28th day of gestation were small and details of the shape of the foetus could not be demonstrated except for heartbeats and suggested that after 40th day details of stomach urinary bladder and umbilical vein of foetus could be demonstrated by ultra sound scanning

Cartee and Rowles (1984) observed a semicircular or C shaped embryo first occurred at gestational day of 22 23 They observed small hyper echoic areas within the lumen on day 10 post breeding and hyper echoic intraluminal embryo averaging 10 mm in length at 17 to 23 days post breeding

According to Shille and Gontarek (1985) the foetal movements and heartbeat could not be identified until days 28 and 35 post breeding respectively During the period of 27 to 30days uterus had an anechoic lumen conta ning a hyper echoic embryo Tavern *et al* (1985) also opined that a reliable pregnancy diagnosis using 5MHz ultrasound was possible in most cases from day 25 of gestation

Concannon (1986) reported that uterine swelling at implantation sites was about one cm in diameter by day 20 and represent localized uterine oedema expansion of the embryonic membranes and early placental development According to Barr (1988) pregnancy in the bitch could be consistently diagnosed between day 24 and day 28 after mating when gestational sacs containing fetal tissue suspended in amniotic fluid were seen By day 28 of gestation generalized foetal movements and foetal cardiac activity could assess fetal viability

Yeager and Concannon (1990) reported that the embryonic mass and heartbeat were first detected at 23 to 25 days after the LH surge The diameter of gestational sac at 20 and 25 days of gestation were found to be one to four mm in diameter and one to four mm in length and 8 2 $\pm$ 0 3 mm in diameter and 20 3$\pm$ 1 1 mm length respectively in beagle bitches

Gestational sacs at 17 to 20 days after LH surge were found to be 1 to 2 mm in diameter and 1 to 4mm in length and the heartbeats could be observed from day 23 to 25 and a focal anechoic area in the head could be visualized from days 27 to 31 (Yeager *et al* 1992) Feldman and Nelson (1996) also found that visualization of the functioning of heart was consistently accompl shed by the 25th day of gestation and stated that differentiation of pregnancy from pyometra and early recognition of pregnancy could be established quickly and safely with ultrasonography

England (1998) found that conceptuses might be first imaged from 15 days after ovulation where they appear as spherical anechoic structures approximately 2mm in diameter and from day 20 after ovulation the embryo could be imaged having 7mm in diameter and 15mm in length But Bhadwal and Mirakhur (2000) reported that the earliest gestation age of foetus was 26 days when it could be easily diagnosed as almost a round anechoic cavity containing hypo to hyper echoic mass By day 30 conceptual swelling was seen as round to oval and contained elongated hyper echoic structure surrounded by anechoic amniotic fluid

According to a study conducted by Johnston *et al* (2001) in 51 pregnant bitches with B mode ultrasonography number of pups could be accurately determined only in 31 8 percent of the pregnancies Zambelli *et al* (2002) reported the earliest ultrasonographic observation of the gestational sac on day 10 after mating while the embryo could be measured only at day 18 by ultra sound scanning Kutzler *et al* (2003) opined that the most accurate predictions of parturition date were obtained when fetuses were measured by day 30

Deka *et al* (2004) found that the foetal viability could be diagnosed as early as 25 days of pregnancy through ultrasonography as indicated by pulsating cardiac movements with 97 62 percent accuracy However the earliest correct diagnosis of pregnancy by ultrasonography was recorded by day 21 after first mating (Arunmozhi *et al* 2005) Kuldeep *et al* (2006) also observed that allantois along with developing fetuses in individual embryonic vesicles could be monitored from day 22 of gestation

2 3 6 Accuracy of Pregnancy Diagnosis by Ultrasonography

Allen and Meredith (1981) found the optimum period for using A mode ultra sound scanning on 32 to 62 days after mating and found that the accuracy of detecting pregnancy was 90 percent against 83 percent in non pregnant bitches However a low accuracy was observed in predicting actual foetal number associated with overlapping fetuses or mistaking them as already counted due to the acoustic artifacts (Shille and Gontarek 1985)

According to Barr (1988) estimation of litter size by ultrasound scanning was not easy but the periods between 28th and 35^{h} day of gestation was the best time for counting the number of fetuses England (1998) found the efficacy of prediction of litter size to be 97 percent in early stages of gestation which dropped to 20 percent during later stages of pregnancy

Examining 66 bitches at various stages of gestation using ultrasonography an overall accuracy in detecting litter size was 96 78 percent and the earliest pregnancy diagnosis with 100 percent accuracy was obtained by 18th day post service Accuracy of estimating foetal viability and litter size was 97 62 and 96 78 percent respectively by 25th day of pregnancy Deka *et al* 2004

2 3 7 Pregnancy Diagnosis by Serum Relaxin Detection

Brenner *et al* (1985) extracted serum relaxin from pooled serum of women in the third trimester of pregnancy The relaxin was absent in the extracts of sera from women in the follicular phase of menstrual cycle and also in the pooled male sera Their findings represent the first demonstration of relaxin like bioactivity in human serum

Steinetz *et al* (1987) found that immuno reactive relaxin was not detectable in plasma of male dogs bitches in anoestrus or pseudo pregnant bitches that had undergone infertile mating whereas immuno reactive relaxin was detectable in plasma in the third or fourth week of gestation n retrievers and beagle bitches Steinetz *et al* (1989) opined that serum immuno reactive relaxin concentration was not detectable before the 4th week of pregnancy and the level of relaxin then rose to maximum level at about 6 7 weeks of gestation

Concannon *et al* (1996) observed that serum relaxin concentration increased from day 26 to 30 and was not detectable in non pregnant dogs and opined that relaxin concentration increased during middle and later stages of gestation Feldman and Nelson (1996) also found that the hormone relaxin was detectable with immuno assay and offered the potential to be a reliable indicator of pregnancy It increased during latter half of pregnancy in bitch but was not detectable at any time in psuedo pregnant bitch

England (1998) observed a rise in relaxin concentration from day 25 with peak values on days 40 to 50 followed by a slight decline before partur tion Pamela (2000) observed that concentration of relaxin increased to detectable

levels in the pregnant bitch approximately 25 days following breeding and peaked on days 40 to 50 Johnston *et al* (2001) also recorded that relaxin was a hormone produced primarily by the canine placenta and was therefore the nearest thing to a pregnancy specific hormone in dog The level of relaxin rose significantly compared to non pregnant dogs beginning at 20 to 30 days of gestation Relaxin was produced by the placenta during pregnancy and was thought to contribute to its maintenance by inhibiting uterine motility (Arthur *et al* 2001)

Bunck *et al* (2002) opined that the mean relaxin content in plasma increased as the pregnancy progressed and decreased slightly during last 2 weeks before parturition The values obtained photometrically or visually correspond to day 24 after ovulation Kustritz (2003) also found that relaxin hormone was produced primarily by the canine placenta and found that serum relax n concentration in pregnant dogs increased significantly compared to the non pregnant dogs which began at 20 to 30 days of gestation and reached peak at mid gestation Hoffmann (2004) suggested that polypeptide relaxin a member of the insulin family was the only pregnancy specific hormone It became detectable during the 4th week of pregnancy and reached a maximum level of 4 to 6 ng/ml at 2 3 weeks before parturition

Alacam *et al* (2005) recorded that all pregnant dogs generated positive data for relaxin on 21 day after last mat ng Relaxin was not detected in abort on mummification cases and hence this could be detected in dogs that had live fetuses as it was highly specific for normal gestational status

Dorsser *et al* (2006) found that relaxin was a pregnancy specific hormone in the queen and was produced by the placenta Rapid bench top kit was found to be accurate in diagnosing pregnancy from 28 days after mating

According to Concannon (2006) the Enzyme Linked Immuno Sorbant Assay (ELISA) was useful to detect pregnancy as early as day 20 to 23 days where as Witness relaxin c often detected pregnancy relaxin as early as day 26 to 30days of gestation

Ozyurtlu (2006) found that immunoreactive relaxin was detected in pregnant bitches between second and fourth weeks of gestation after the LH surge reached the peak levels two to three weeks prior to parturition (more than 3 0ng/ml) It was usually detected between 21 and 26 days of pregnancy Qualitative relaxin test results were positive only n the pregnant bitches and were negative in overt and covert pseudo pregnant bitches

Romagnoli (2006) opined that relaxin was a key hormone of pregnancy both in the bitch and the queen as it rose between the third and fourth week of gestation and reached peak at 2 to 3 weeks pr or to parturition It was non detectable in males in non pregnant females and during stages other than pregnancy or the puerperium

2 3 8 Accuracy of Pregnancy Diagnosis by Relaxin Assay

Feldman and Nelson (1996) reported that although relaxin assay was apparently sensitive and reliable its concentrat on was not enough to detect pregnancy earlier than mid gestation But Pamela (2000) observed that the relaxin ELISA was specific and sensitive for detecting pregnancy in bitches without the occurrence of false posit ve results Additionally the relaxin assay was quite effective at distinguishing pseudo pregnancy from actual pregnancy since that hormone was absent in psuedo pregnant bitch

Johnston *et al* (2001) found that false negatives for pregnancy relaxin occurred when bitches were tested as early as 21 days of gestation Kustritz (2003) also reported that serum relaxin concentration in pregnant dogs increased significantly compared to the non pregnant dogs which began at 20 to 30 days of gestation

Alacam *et al* (2005) opined that relaxin hormone levels could be assayed using Repro Chek or Witness relaxin tests for the detection of pregnancy and viability of foetus It was practical in bitches by which normal pregnancy could be differentiated from false pregnancies

Concannon (2006) found that false positive results occurred in cases of foetal resorption or with retained placental tissue Similarly Ozyurtlu (2006) suggested that relaxin measurement might provide useful information about whether or not a pregnancy had been established or if foetal death and resorption had occurred Romagnoli (2006) also opined that relaxin kit would be very helpful to establish if foetal death and resorption had occurred during pregnancy

2 4 HAEMATOLOGICAL STUDIES

2 4 1 Haemoglobin (Hb)

According to Saror *et al* (1979) normal value of haemoglobin in dog was 14 2+/ 1 6gm/dl Benjamin (1985) reported the value of haemoglobin in the range of 12 to 18g/dl in non pregnant bitches According to Prabhakaran *et al* (1996) hemoglobin content and packed cell volume did not show much variation between pregnancy and non pregnancy Thou (1999) had given mean values of haemoglobin on days 21 to 25 30 to 35 and 45 to 50 in pregnant as 12 47±0 23g/dl 11 91+ 0 22g/dl and 11 0+0 18g/dl respectively and that in non pregnant were 14 19±0 25g/dl 14 03+0 27g/dl and 14 23+0 27g/dl respectively and reported a significant variation in haemoglobin content of pregnant and non pregnant dogs Asha (2005) recorded haemoglobin concentration at 20 to 30 31 40 and 41 to 65 days of gestation were 11 73+0 18 10 2±0 2 10 69+0 2g/dl respectively and in non pregnant was 12 37+0 28 g/dl which was higher than pregnant dogs Significant variation in haemoglobin values between pregnant and non pregnant was also observed

2 4 2 Packed Cell Volume (PCV)

Saror *et al* (1979) reported a normal mean value of PCV as 42 45% in dogs Benjamin (1985) recorded 37 to 55% with an average value of 45% for packed cell volume in apparently normal non pregnant dogs England (1998) opined that packed cell volume were less than 40% at day 35 and below 35% at term Thou (1999) found a decrease in PCV as pregnancy progressed and found the mean values on 21 to 25 30 to 35 and 45 to 50 in pregnant bitches as 41 6+0 33 39 33±0 33 and 35+0 577% respectively and that in non pregnant bitches were 44 5+0 289 44 75+0 75 and 45 25+0 25% respectively in Alsatian bitches Asha (2005) recorded PCV values in pregnant bitches at 20 30 31 40 41 65 days of pregnancy were found to be 39 83±0 72 37 7+0 82 and 36 88+0 8% respectively whereas the values in non pregnant doge were 45+1 15% Significant variations in packed cell volume values before and after conception were also recorded

2 4 3 Erythrocyte Sedimentation Rate

Benjamin (1985) and Sastry (1989) noted 5 to 25 mm/hr as the normal range of erythrocyte sedimentation rate in dogs and stated that erythrocyte sedimentation rate rised in female dogs during pregnancy Henry (1996) opined that increase m ESR was because of the change in erythrocyte/plasma ratio which favoured the rouleaux formation independent of changes in the concentration of plasma proteins Thou (1999) found an increase in the ESR rate ranging between 8 5 to 19 33mm/hr and it was higher when compared to non pregnant bitches ranging between 5 6to5 88 mm/hr Asha (2005) recorded the ESR rate in pregnant were 12 03+0 97 17 62+1 11 and 17 07+1 09mm/hr at 20 30 31 40 41 65 days of gestation respectively whereas in non pregnant an mals it was 25±1 57 Statistical analysis revealed significant variation in ESR between pregnant and non pregnant animals

2 5 GESTATION LENGTH

According to Sokolowski (1980) the average gestation length for the bitch was approximately 62 days However viable fetuses were whelped which followed a gestation of 58 to 66 days

Concannon *et al* (1983) conducted studies in a beagle colony in which apparent gestation length was estimated as the interval from the day of first mating to the day of parturition which ranged from 57 to 72 days and averaged 65 3+0 2 days and the gestation length in the dog was expressed as the interval from an initial or a single mating to parturition with an average of 62 64 days Johnson (1986) also stated that the gestation length in bitch varied from 58 to 72 days after breeding date as ovulation occurred at variable and unpredictable times during behavioural or cytologic oestrus because canine sperm could maintain its ability to fertilize for at least 4 to 6 days in the female genital tract

Kahn (1994) opined that breeding date in bitches differed considerably from the ovulation date and thus made it difficult to accurately establish gestational age in dogs According to Feldman and Nelson (1996) the whelping date was l kely to be 56 to 58 days after the first day of diestrous as determined by vag nal cytology The authors also opined that, this clearly influence calculation of probable due date of whelping and thus influence the results of pregnancy diagnosis

In case of breeding during estrous diestrous was replaced by the period of pregnancy which showed a rather constant gestation length averaging 63 + 2 days which varied between 57 72 days due to long period of receptivity at oestrus and the extended period of sperm survival in the female gen tal tract (Hoffmann *et al* 1999)

Concannon (2000) recorded that using the day of mating or insemination parturition m ght occur as early as 56 days or as late as 68 days in bitches A large variation in apparent gestation length could be encountered when counting from multiple matings or the last of multiple matings However Purswell *et al*

(2000) stated that calculating the expected whelping dates from breeding dates was the most common but also the most inaccurate method because of the var ability in length of oestrus in bitches

2 6 LITTER SIZE

Bondestam *et al* (1984) stated that the accurate estimation of litter size by ultrasound scanning was proved to be difficult, especially in large breeds of dogs and reported a figure of 40 percent accuracy at 29 days after mating and 83 3 percent accuracy from 50 days to term

Concannon (1986) reported that mean litter size varied among breeds ranging from ten pups in blood hounds and Pekingese to fewer than three pups per litter in pomeranians In most breeds the mean litter size was between four and eight Schroeder and Smith (1995) also recorded a litter size of two to nine pups with an average of 5 43 pups in a study of 30 German shepherd bitches However Gradil *et al* (2000) stated litter size depend on several factors and concluded that small sized breeds had two to four medium sized breeds four to seven and large sized breeds had six to ten pups

2 7 GESTATIONAL ACCIDENTS

Allen *et al* (1989) reported a case of hydrops foetalis diagnosed in a near term bitch using real time ultrasonography The affected foetus was identified by the presence of intra thoracic and subcutaneous fluid

England (1992) found the components of embryonic resorption as reduced volume and changes in echogenisity of embryonic fluid loss of embryonic mass and absence of heartbeat Following resorption the uterus was moderately hypo echoic in appearance similar to the picture after parturition Similarly Ettinger and Feldman (2000) reported the incidence of early embryonic death and spontaneous abortion in bitches According to them resorption of the conceptuses occurred until day 45 of pregnancy without any noticeable signs

Bhadwal and Mırakhur (2001) reported a case of pregnancy at 35th day of gestatıon that was about to abort The authors descrıbed ıt as an echoıc area wıth hyper echoıc mass ınsıde No foetal movement or heartbeat could be observed

Accordıng to Hopper *et al* (2004) out of 161 canıne pregnancy dıagnosed by ultrasonography ıncıdence of foetal resorptıon abortıon stıllbırth neonatal mortalıty were 7 4 8 4 8 15 and 9 6 percent respectıvely

Materials and Methods

3 MATERIALS AND METHODS

3 1 SELECTION OF CASES

Animals for the study consisted of 45 apparently healthy bitches selected at random from those presented for detecting optimal time for breeding at University Veterinary Hospitals of Kokkalai and Mannuthy during the study period The bitches brought for breeding advice were first subjected to clinco gynaecological examination and exfoliative vaginal cytology for finding optimal breeding time Out of this ten animals selected at random were subjected to different methods of pregnancy diagnosis at different periods of gestation The body weight of these animals was recorded and blood was taken for routine haematological studies *viz* haemoglobin (Hb) packed cell volume (PCV) and erythrocyte sedimentation rate (ESR)

3 2 ASSESSMENT OF OPTIMAL BREEDING TIME

3 2 1 Clinico gynaecological Examination

Bitches presented were subjected to clin co gynaecological examination and complete breeding history was collected All the bitches were examined in detail for intensity of vulval oedema (low medium and high) nature of proestral discharge (sanguineous sero sanguineous and straw coloured) and any abnormalit es of external genitalia (Plate 1)

3 2 2 Exfoliative Vaginal Cytology (EVC)

3 2 2 1 Preparation of Vaginal Smear

Vaginal discharge was collected by the technique described by Allen and Dagnell (1982) The an mal was controlled in standing position and a sterile pipette with adaptor and syringe at distal end was carefully introduced into the vag na directing the pipette cran o dorsally to the vestibule and at the vestibulo vaginal

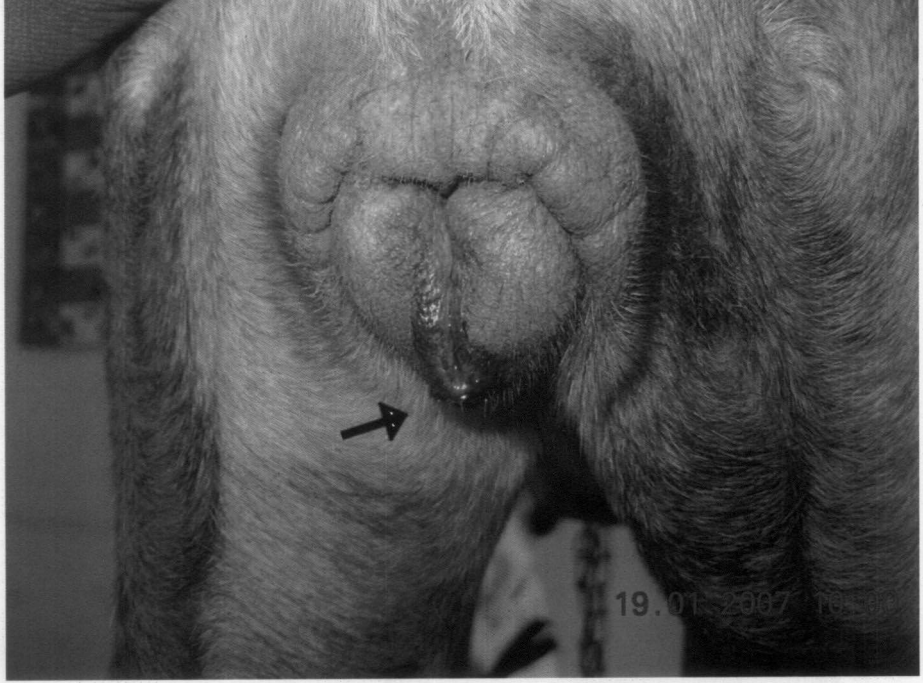


Plate: 1 Proestrum bitch showing oedematous swelling of the perineum and vulva. The vaginal discharge of sero-sanguineous fluid

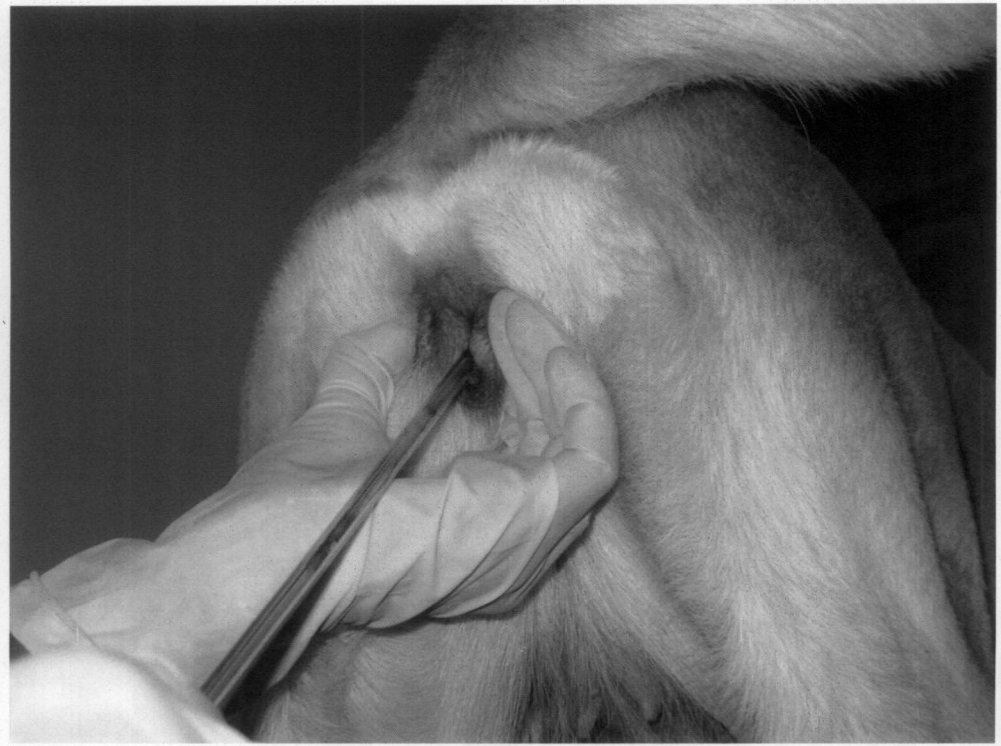


Plate: 2 Collection of vaginal discharge using a sterile pipette for performing exfoliative vaginal cytology. Note the cranio-dorsal direction of pipette

junction and then redirected cranially to reach the anterior vagina (Plate 2) The fluid was aspirated and a small drop of discharge was kept on the slide a thin smear was prepared out of it and then it was air dried

3 2 2 2 Staining of Vaginal Smears

The vaginal smears were stained using **Modified Wright Giemsa stain**

Composition of Modified Wright Giemsa stain

Wright stain powder	300mg
Giemsa stain powder	30mg
Methanol	100ml

Procedure

1 Prepared the vaginal smear on clean grease free glass slide and air dried

2 Covered the smear with modified Wright Giemsa stain and allowed to act for 30 seconds

3 Flooded the slide with distilled water mixed by blowing and allowed to act for 30 seconds

4 Washed with water dried and examined under high power of microscope (40 X 10X)

3 3 TYPES OF CELLS ENCOUNTERED IN EVC

3 3 1 Epithelial Cells

They are classified into three major categories *viz* parabasal cells intermediate cells and superficial cells

3 3 1 1 Parabasal Cells

These are the smallest epithelial cells seen in smears and are round or oval in shape The nucleus occupies about 45 to 90 percent of the cells

3 3 1 2 Intermediate Cells

These include cells of varying size and types which represent all stages of maturation between parabasal and fully mature superficial cells The cells become more angular enlarge and flatten as they mature The relative size of the nucleus decreases as they mature The small intermediate cells are small and polygonal with a relative large nucleus

3 3 1 3 Superficial Cells

They are large polygonal cells with irregular or folded borders with or without nucleus Based on the nuclear characteristics there are four types of superficial cells

- Large polygonal dead cells with irregular borders without any nucleus
- Large polygonal cells with intact nuclear membrane
- Large polygonal cells with small remnant of nuclei
- Large polygonal cells with pyknotic nuclei

3 3 2 Anuclear Cell Index

Anuclear cell index Number of anuclear cells / Total number of epithelial cells X 100 Breeding of bitches at 72hrs interval was advised based on history clinico gynaecological examination and anuclear cell index (more than 80 percent cells anuclear)

3 4 HAEMATOLOGICAL STUDIES FOR DETECTION OF PREGNANCY

3 4 1 Site for Blood Collection

Blood (5ml) was collected from cephalic vein or saphenous ve n on the day of breeding as well on different gestational ages Sodium citrate was used as the anticoagulant (3 8% at the rate of 1ml/9ml blood) Then it was used to estimate haemoglobin (Hb) packed cell volume (PCV) and erythrocyte sedimentation rate (ESR) Serum was separated from 2ml of blood by centrifuging at 3000 rpm for 30mts The serum thus obta ned was subjected to qualitative relaxin detection by rapid immuno migration technique

3 5 PREGNANCY DIAGNOSIS BY DIFFERENT METHODS

Out of the bitches advised breed ng based on exfoliative vaginal cytology ten were selected randomly for pregnancy diagnosis by different methods

Group1 — Ten an mals belong ng to this group were subjected to abdominal palpation transabdom nal ultrasonography and rapid immuno migration test (Witness Relaxin us ng the combination of anticanine relaxin antibodies and serum resulting in the formation of purple band in both test and control wells) between 16 to 20 days post breed ng

Group 2 — The same animals were mon tored for pregnancy by abdominal palpation transabdominal ultrasonography and rapid immuno migration test but at different gestat on age ranging from 21 to 24 days

Group 3 The same animals were reviewed on third visit for pregnancy confirmation by abdominal palpat on transabdominal ultrasonography and rapid immuno migration test between 25 to 30days of gestation

During each visit body weight was recorded Blood samples were collected for estimating haemoglobin (Hb) packed cell volume (PCV) and erythrocyte sedimentation rate (ESR)

Those animals which are pregnant were monitored till whelping and the conception rates were recorded Accuracy in diagnosing pregnant and non pregnant status was compared with the actual whelping data and the results were tabulated accordingly

3 5 1 Transabdominal Palpation

The method described by Sokolowski (1980) was adopted in this study The bitch was controlled in standing position grasped the abdomen gently by applying gentle pressure up toward lumbar spine and then gently bringing the fingers together allowing the abdom nal viscera to slip through the fingers to locate pregnant uterus and by identification of discrete round or oval swelling of a size expected to the respective dates in the uterus after mating were assumed as positive (Plate 3)

3 5 2 Ultrasound Scanning

Ultrasound equipment (Honda electronics HS 2000 vet) that produces two dimensional gray scale real time images was used for ultrasound scanning The frequency used was 5 to 7 5 MHz An mals were positioned n dorsal recumbency and prepared the mid ventral area from the pubis to just cranial to umbilicus Coupling medium (Methyl Cellulose) was applied to the skin and also to the transducer probe to assure good acoustic transmission Both sagital and transverse scan planes were used For scanning the abdomen was considered as

Plate: 3 Transabdominal palpation

Plate: 4 Transabdominal ultrasound scanning

two rec ons one r ght of and the o her left of the ed al plane Fa l ec on scanned separately and the results were non tored (Pla e 4)

3 5 3 Canine Pregnancy Test Kit Witness Relaxin

3 5 3 1 Test Principle

W tness Relax n s a s mple test based on Rap d I m no M gra (RIM) techn que us ng the co nb nat on of ant can ne relax n ant b d q ckl dent fy th s hormone n b olog cal sa nple (serum or pla a) Iron b tch Sens t zed collo dal gold part cles b nd to relax n molecules pr se n sample The complexes m grate along a n trocellu ose str p and are hen cap on a sens t zed react on l ne where ts accumulat on causes the lor nat on clearl v s ble purple band n both test and control ells (ndo # w ndo v#3) A control band at the end of the read ng ndo v ensure hat th was perfor ned correctl (Plate 5)

K t contents (Plate 6)

5 pouches each conta n ng l test dev e a id des ccant

- 1 buffer dropper bottle (2ml)
- Instruct ons for use
- 5 p pettes

3 5 3 2 Test Procedure

San ple appl cat on

- Tear opened the pouch and placed the tes dev ce on a flat h r z a s rface for the durat on of tl e test

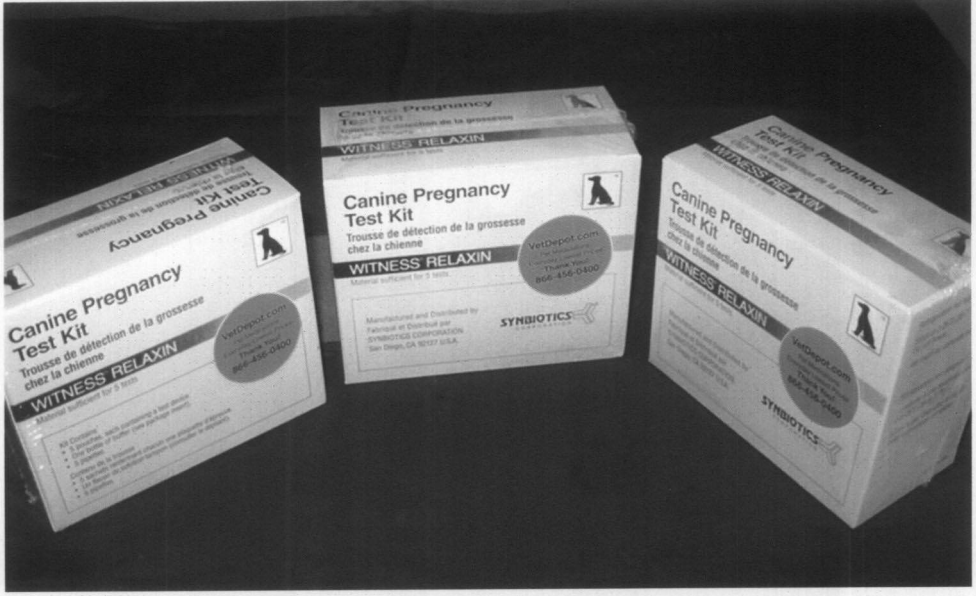


Plate: 5 Canine pregnancy test kit - Witness relaxin. For serum relaxin detection by Rapid immuno-migration technique.

Plate: 6 Witness relaxin - Kit contents

. test devices

. buffer

. leaflet

- Squeezed the prov ded p pette near the scaled end l r ed th j end of the p pette nto the ser sa nple Released tl c press r nt y dra v up a small amount of serum sample nto the p pett
- Held the p pette vert cally and transferred vo drop ol san p sample well v ndo v #1

Buffer d spens ng

T o drops of buffer as added o he vell #1

Read ng test

- W a ted for ten m nutes observed for the presence or absenc o p p band n read ng v ndow #2 and #3(both test and t l
- Sample res lts are read n w ndo v # (test) The control band a ad w ndow #3 (control)

3 5 3 3 Results

Val dat on

Val d test Test s val dated f a purple band as p esent n he ad v ndow #3

Interpretat on

Positive for relaxin One purple band n read n ndo #? th on p p band n window #3 (n both test and control w ndo vs)

Negative for relaxin No band n read ng v do #2 (test) h on p p band n v ndow #3(control)

Invalid test No purple band n control ndo #

Accuracy n pregnancy stat s and l tter s ze as recorded a d co npared to actual help ng data obta ned by regula follo p Ges a o l as calculated from the f rst day of breed ng to he p ng date

3 6 STATISTICAL ANAI YSIS

Pa red l test vas conducted to test the s gn f canc b ee d fferent var ables The results were analyzed as per Snedec r a d Co l (198ɔ)

Results

4 RESULTS

Exfoliative vaginal cytology was done in 45 bitches that were brought to University Veterinary Hospitals of Kokkalai and Mannuthy during the course of study Detailed clinico gynaecological examination and exfoliative vaginal cytology were done in all the animals to assess the optimal breeding time Out of this ten animals of different breeds of medium s ze were selected at random for pregnancy diagnosis at different periods of gestation *viz* 16 to 20 21 to 24 and 25 to 30 days of gestation The body weight of these animals were recorded periodically and blood was collected for haematological evaluation

4 1 OPTIMAL BREEDING TIME

In the study conducted in ten animals it was observed that major ty of them was presented at the time of mid proestrum with high intensity of vulval oedema and sero sanguineous discharge from vulva Many of them resisted digital manipulation of vulva during this period

It was observed that the discharge decreased at the time of late proestrum together with mild degree of vulval oedema in six animals but in the other four bitches sero sanguineous discharge with a medium degree of vulval oedema was observed Exfoliat ve vaginal cytology findings are shown in Plates 7 to 12 Breeding advice was given based on anuclear cell index reaching more than 80 percent (Plate 10)

4 1 1 Pregnancy Percentage Based on Breeding

Out of ten animals brought for pregnancy diagnosis where breeding was advised based on the anuclear cell ndex and symptoms of oestrum all the ten animals became pregnant (100 percent) Among 35 animals where breeding was advised based on the symptoms of heat signs alone 20 animals became pregnant (57 14 percent) The results were furnished in Table 1

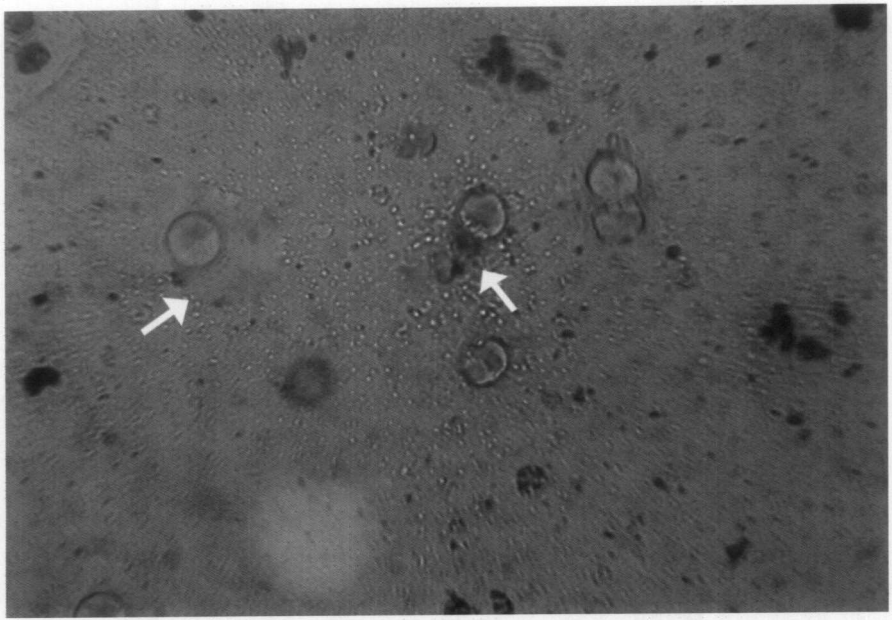


Plate: 7 Exfoliative vaginal cytology illustrating small intermediate cells with few amount of cell debris. Wright-Giemsa stain-Magnification 400X

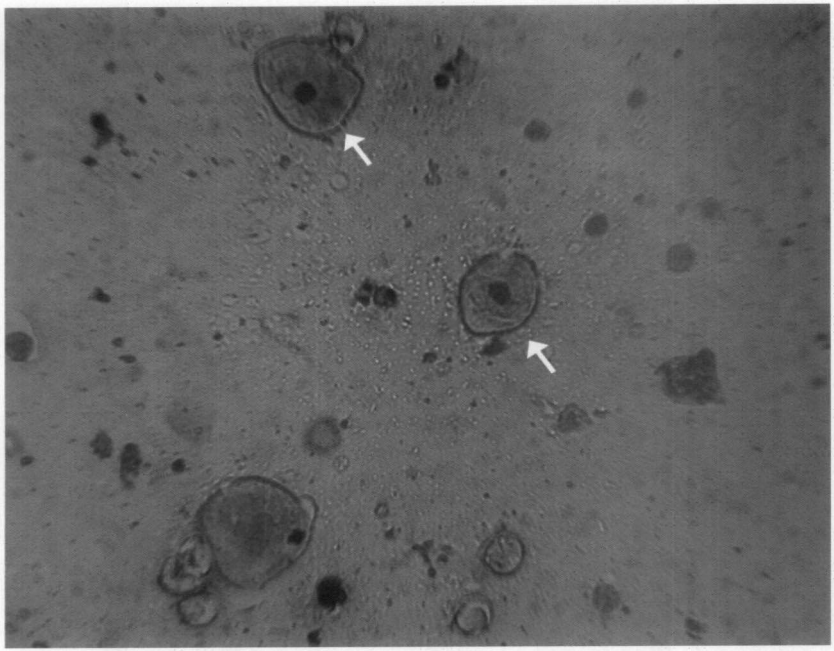


Plate: 8 Exfoliative vaginal cytology revealing large intermediate cells suggestive of mid proestrum. Wright-Giemsa stain-Magnification 400X

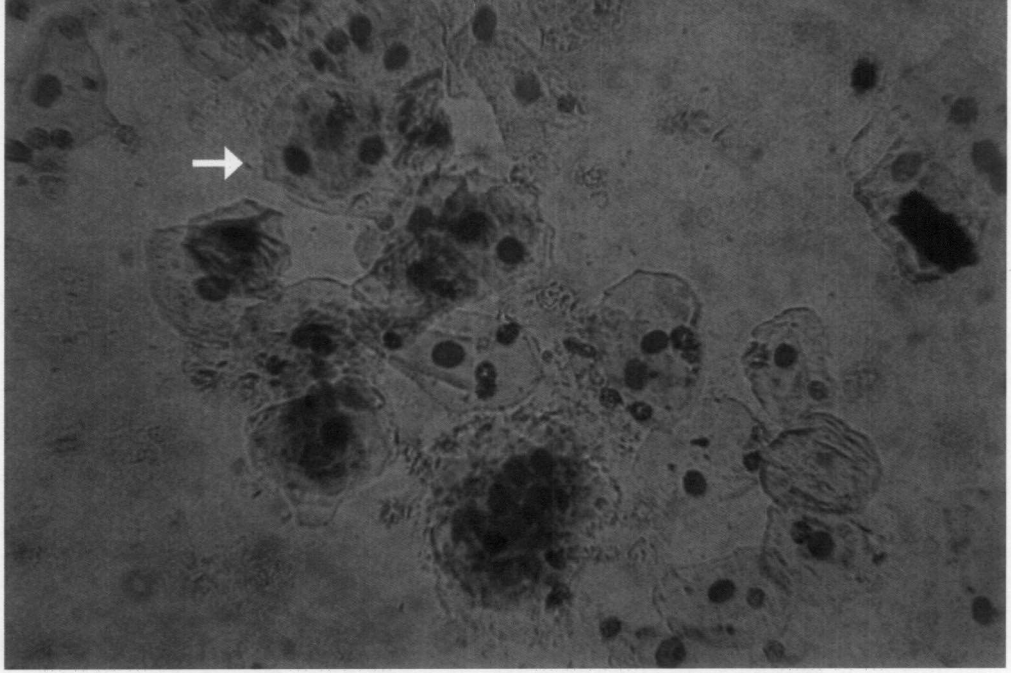


Plate : 9 Exfoliative vaginal cytology revealing large number of superficial intermediate cells. Smear suggestive of late proestrum. Wright-Giemsa stain, Magnification 400X.

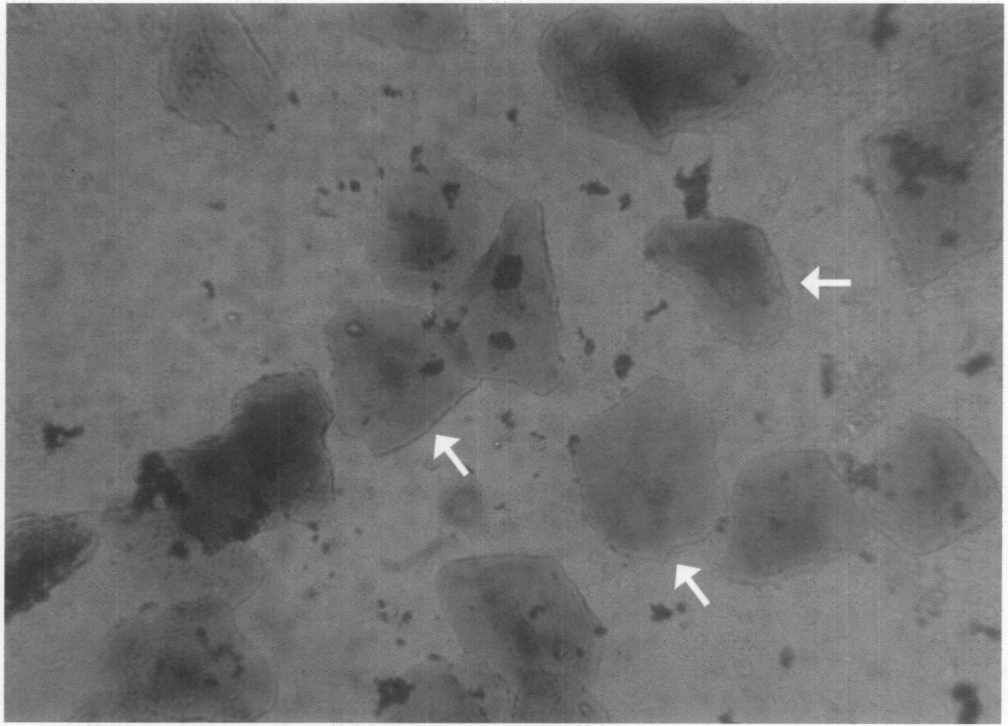


Plate : 10 Exfoliative vaginal cytology revealing anuclear superficial vaginal cells, no red blood cells or netrophils with clear back ground. This finding is classic for estrus in bitch. Wright-Giemsa stain, Magnification 400X.

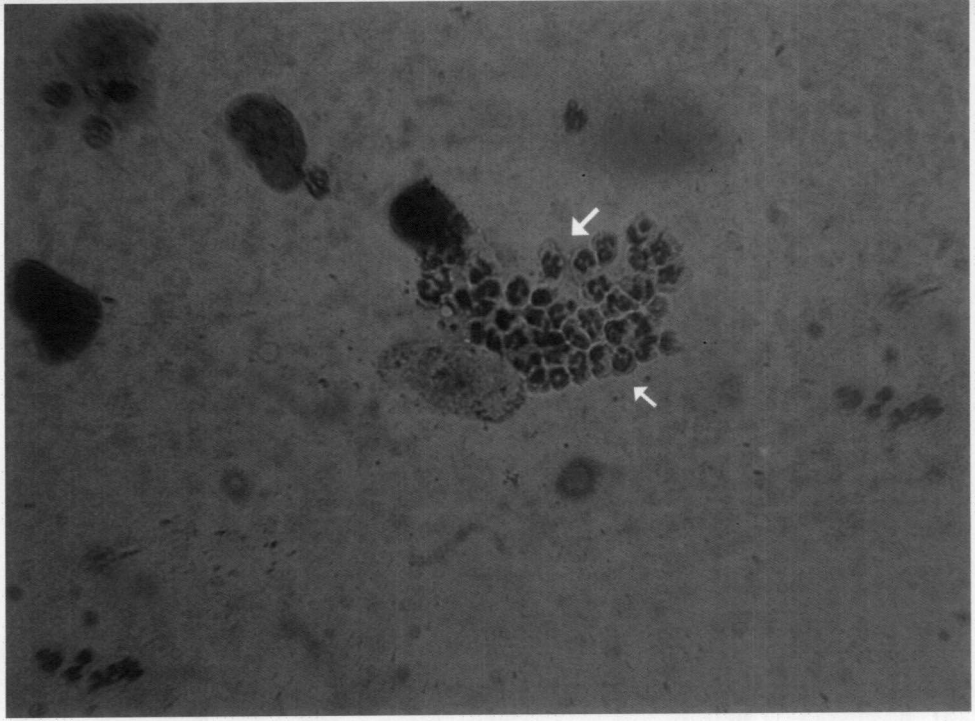


Plate : 11 Vaginal smear from a bitch revealing large number of neutrophils, suggestive of vaginitis. Wright-Giemsa stain, Magnification 400X.

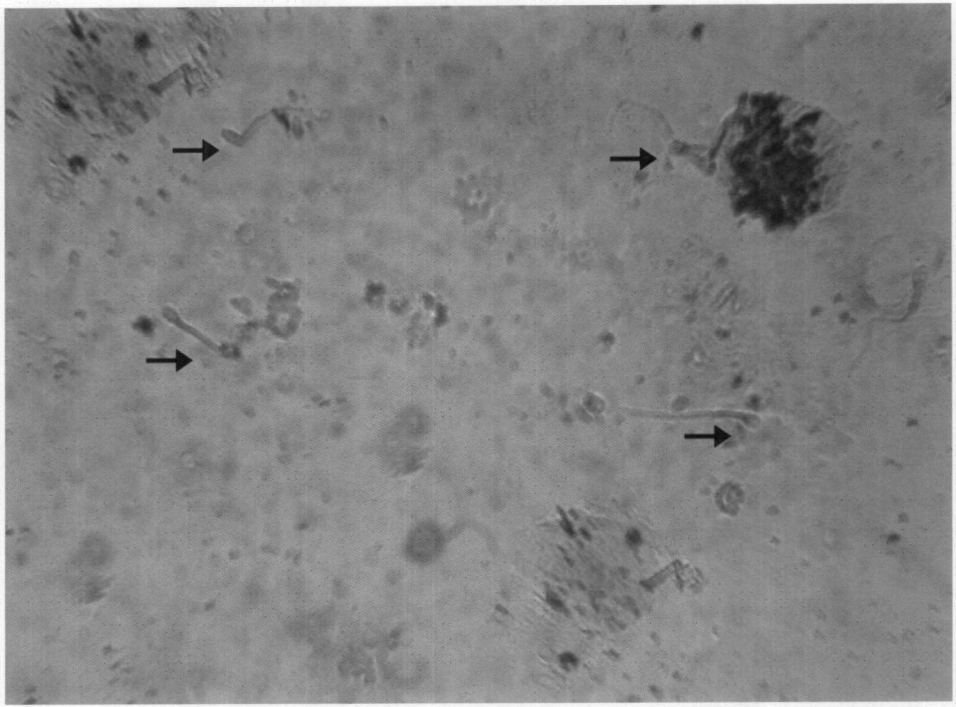


Plate : 12 Vaginal smear from a bitch that had bred earlier. Note the sperm present in the smear confirming that breeding has taken place. Wright-Giemsa stain, Magnification 400X.

Table 1 Compar son of co ncept on rate of an n als bred based n cl oL examination and exfoliat ve vag nal cytology w tl tho br d b l s ɛns

Meth od of breeding	Number of animals examined	Number l animals ec neceived	C cep ti t
Bre l n l d o exl l a ag lc lo_ rd l s	10	1	
Bas d n l s ɛ alo			

4 2 PREGNANCY DIACNOSIS AT DIFFERENT GESTATIONAL AGE

The resul s of p egnancy d agno s by d ffe nt c hod f Table and Plates 13 to 22

4 2 1 At 16 to 20 Days Post Breeding

When transabsdom al palpat on a car d o a 6 da found hat n only t vo an nals sl ght u er nc d s e on ould l n vas recorded as doubtf l and seven er categor zed unde n g

When they vere subjected to ransabdo nal ltras o pl an mals e nbryon c ve cles were observed as spher cal t u re l cons stency No foe us or foetal nen b anes could be app cc a ed a l o an mals ere re orded as doubtful and h ee e c a d negat ve

Whe the seru san ples ere subjected f r r lav n d e n b n nuno m grat on te l n q c n f ve sa npl p rple a ob d a the te t (v ndow #?) a d control (w ndo v #3) sug ges g pos t ve f p In th ee sa nples a doubtful resul as obta ned here a fa n p p e observed n test vell and adv sed a re exa n nat on afte f ve da s l no purple l ne vas obta ned when exa ned at 16 da and a c o neg at ve

4 2 2 At 21 to 24 Days Post Breeding

When s bjected to abdo n nal palpat on c at o a a apprec ated n f ve an als as a ound truct re four an mal e r c d doubtful and one an mal wa categor zed as negat ve

On transabdom nal ltras nog aphy a h p r e l o c tral n as observed n c gh an als Fl u ne wall r ound g he a as h pe echo c con pared th he res of the t ne ho n S la

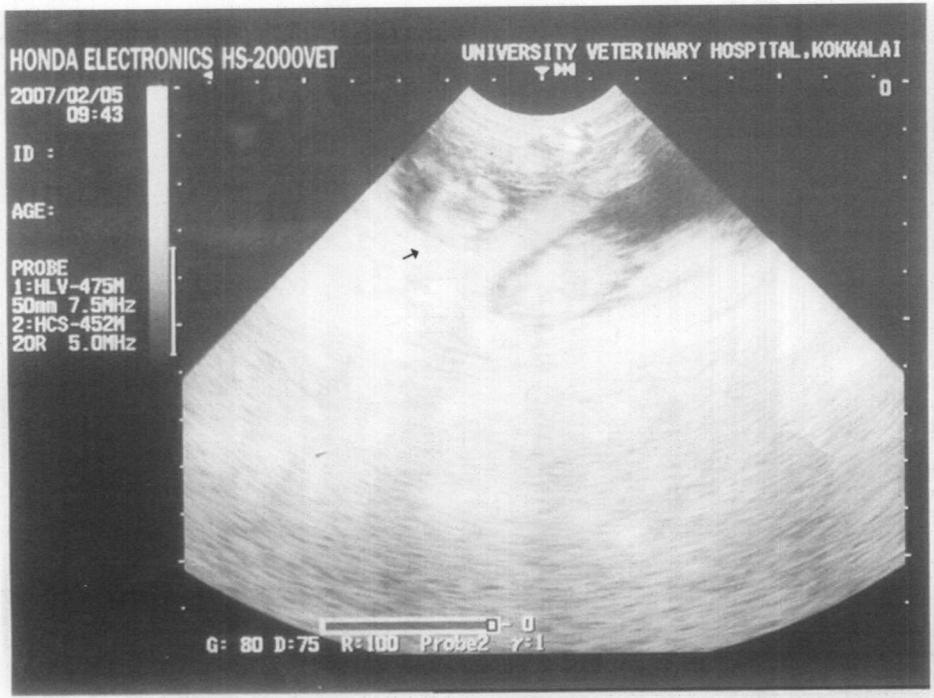


Plate: 13 Sonogram of uterus of a bitch at 20 days of gestation. Foetus as well as foetal membrans are non detectable.

Plate: 14 Sonogram of uterus of a bitch at 23 days of gestation. The foetal sac could be seen as hypoechoic lumen surrounded by an echoic uterine wall. Foetus could be visualized at this stage.

Plate : 15 Sonogram of uterus of a bitch at 25 days of gestation. The foetal sac could be seen as a hypoechoic lumen.

Plate : 16 Sonogram of uterus of a bitch at 28 days of gestation. Embryo together with placenta could be observed at this stage

Plate : 17 Sonogram of uterus of a bitch at 35 days of gestation left side - B mode, right side - M mode representing foetal heart beats

Plate : 18 Sonogram of uterus of a bitch at 50 days of gestation Vertebrae were observed as whitish hyperechoic areas arranged in a segmented pattern.

Plate : 19 Sonogram of uterus of a bitch at 60 days of gestation. Foetal heart, urinary bladder and vertebrae could be observed in this plate.

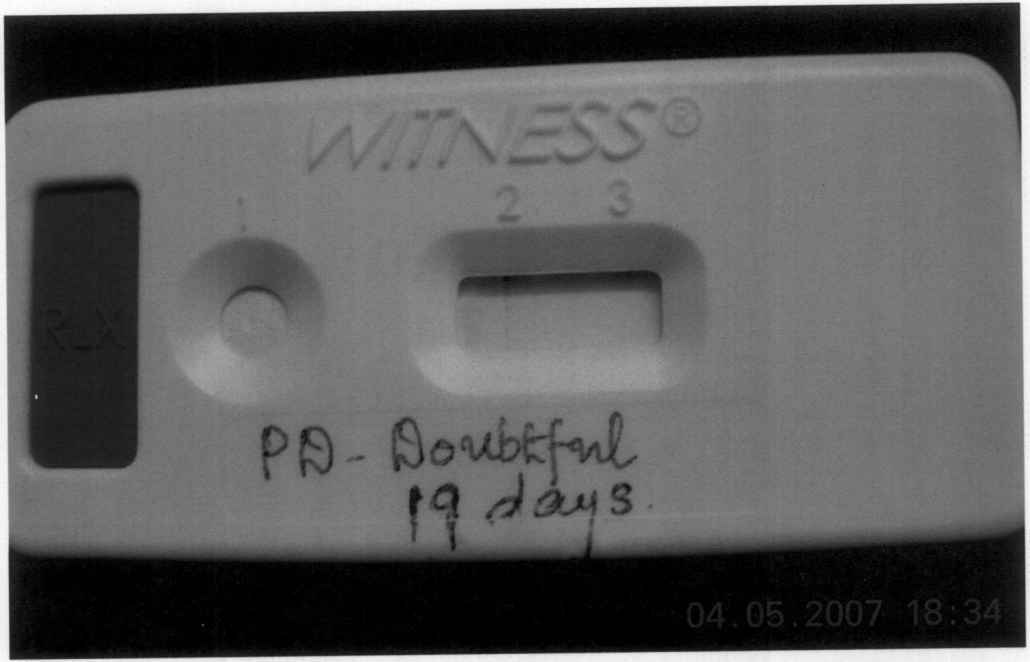


Plate : 20 Serum relaxin test-Doubtful for pregnancy.
By Rapid immuno-migration technique.

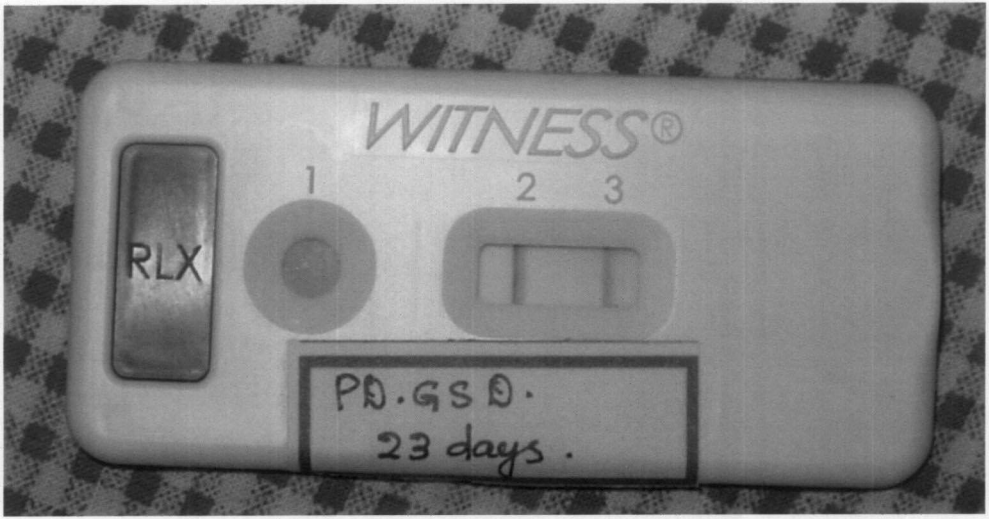


Plate : 21 Serum relaxin detection by Rapid immuno-migration technique. Purple line at window # 2 & #3 (test & control) indicates positive for pregnancy.

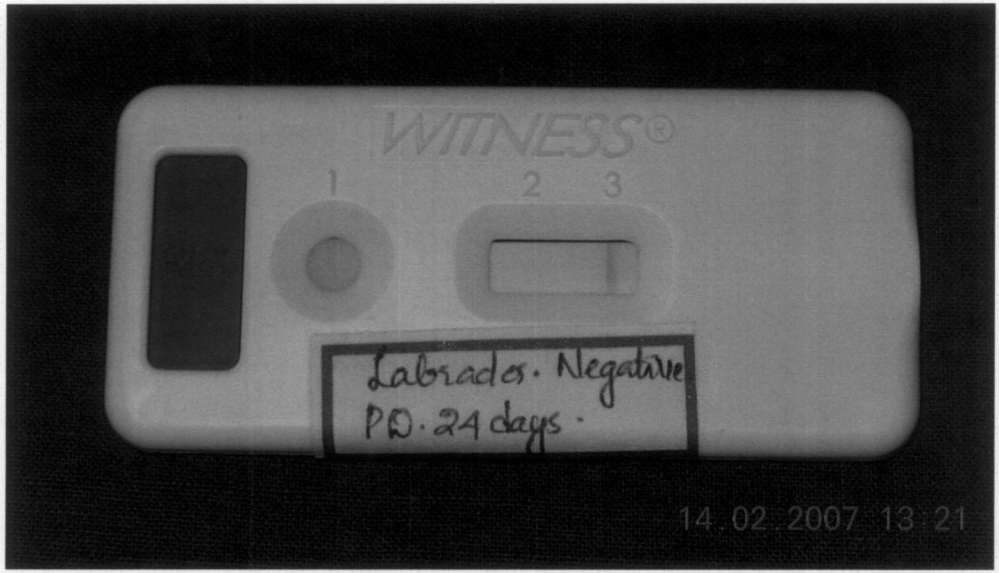


Plate : 22 Serum relaxin detection by Rapid immuno-migration technique. Purple line at window # 3 (control) indicates negative for pregnancy.

Table 2 Comparison of percentage accuracy for abdominal palpation ultrasound scanning and relaxin detection as different methods of pregnancy diagnosis

Group	Post breeding days	Abdominal palpation			Ultrasono graphy			Relaxin detection			Percentage accuracy (%)		
		P	D	N	P	D	N	P	D	N	AP	US	Rlx
I	16 20	2	1	7	5	2	3	5	3	2	20	50	50
II	21 24	5	4	1	8	2		10			50	80	100
III	25 30	7	1	2	10			10			70	100	100

P Positive
D Doubtful
N Negative

AP Abdominal palpation
US Ultrasound scanning
Rlx Relaxin detect on

animals cardiac activity was monitored as flickering heart beats Two animals were recorded as doubtful

When relaxin assay was done in all the ten samples purple line was obtained in both test (window #2) and control (window #3) suggestive of pregnancy was obtained

4 2 3 At 25 to 30 Days Post Breeding

By abdominal palpation technique at 25 to 30 days post breeding foetus could be appreciated as tense distinct uterine swellings like strings of pearl in seven animals In one animal a doubtful result was obtained and two animals were categorized as negative

By ultrasonography presence of embryo could be visualized as an anechoic structure with hypo echoic wall Foetal heart beats as well as placenta could be observed at this stage in all the ten animals

In relaxin test purple line was obtained in all the ten animals in both the test and control confirming pregnancy

4 3 PERCENTAGE ACCURACY OF DIFFERENT METHODS OF PREGNANCY DIAGNOSIS

4 3 1 Transabdominal Palpation

The earliest positive result obtained by abdominal palpation was between 16 to 20 days post breeding but the accuracy was only 20 per cent and more negative cases were recorded at this period (80 per cent) When palpation was done in between 21 to 24 and 25 to 30 days post breeding the accuracy obtained was 50 per cent and 70 per cent respectively (Fig 1)

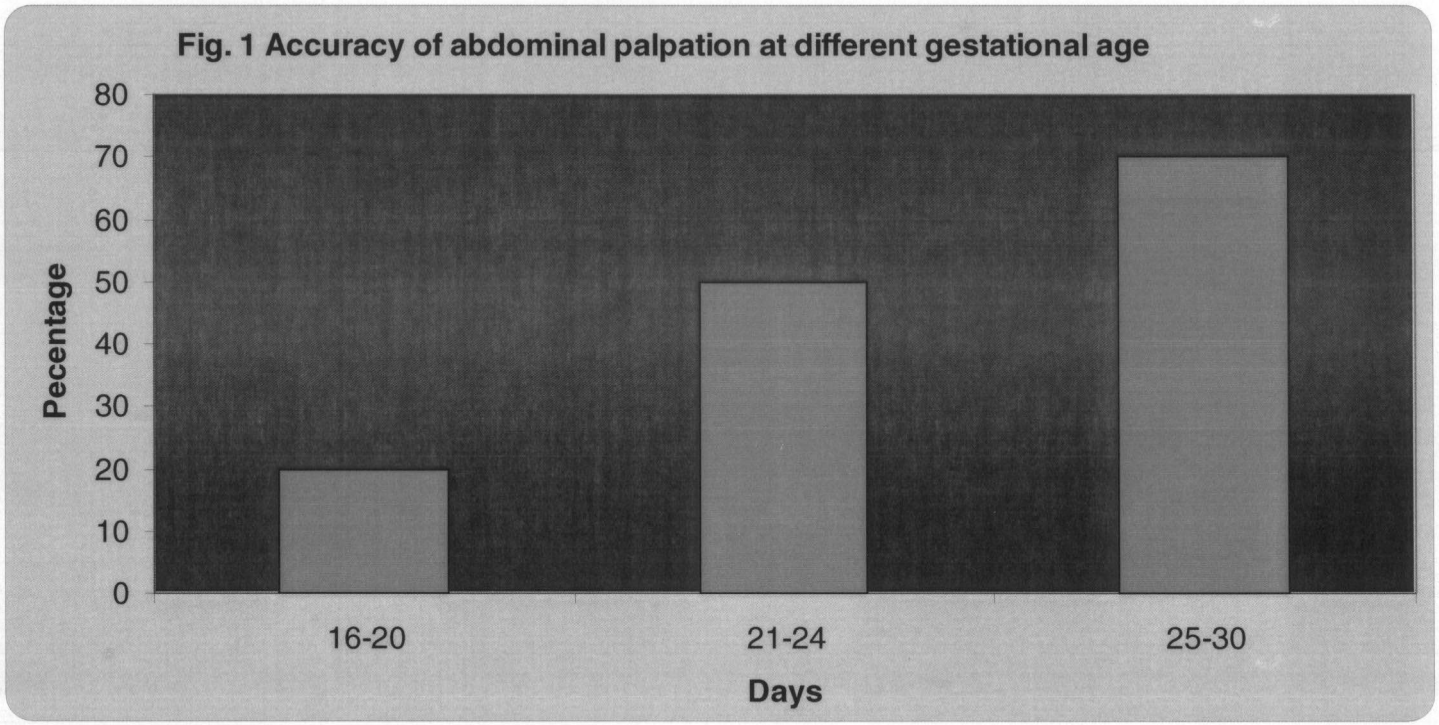


Fig. 1 Accuracy of abdominal palpation at different gestational age
Pecentage
80
70
60
50
40
30
20
10
0
16-20
21-24
25-30
Days

4 3 2 Trans abdominal Ultrasonography

By ultrasound scann ng he perc tage accurac of pre nan d at 16 o 20 days vas 50 per cent h ch mproved o 80 percen d 0) at 21 24 and 25 30 da s post breed ng respect vely F etal hear b obser ed n all the pos t ve cases fro 24 days of es at on (F)

4 3 3 Serum Relaxin Detection

The earl est pos t ve result obta ned f r seru n relax d c obta ned at 20[h] da post breed ng and the percentage accu a a per od as aga nst 100 per cent at 21 o 4 and 25 o 30 da s ol e at (I

4 4 HAEMATOLOGICAL STUDILS

4 4 1 Haemoglobin (Hb)

Hae noglob n conce ntrat on at da 0 16 to 0 21 to 4 and I of estat o ere found o be 11 56 0 27 10 88 0 31 I 4 8 77 0 g dl respect vely The le el of haemoglob n as found e l as pregnancy advanced (Tabl and F 4) Sta cal a a s s gn f cant reduct on n hae noglob n concentrat on at d fferent a a (P<0 01)

4 4 2 Packed Cell Volume (PCV)

Packed cell volume values ere 34 66 0 9 0 77 0 94 8 26+0 94 percent at da 0 16 to 20 21 to 24 and 5 o 30 dav ot estat vas gradual reduct on n the PCV values dur n d ffe ent ta es f (Table 3 and F g 5) Stat st cal analys s revealed that th a at s g f cant (P<0 01)

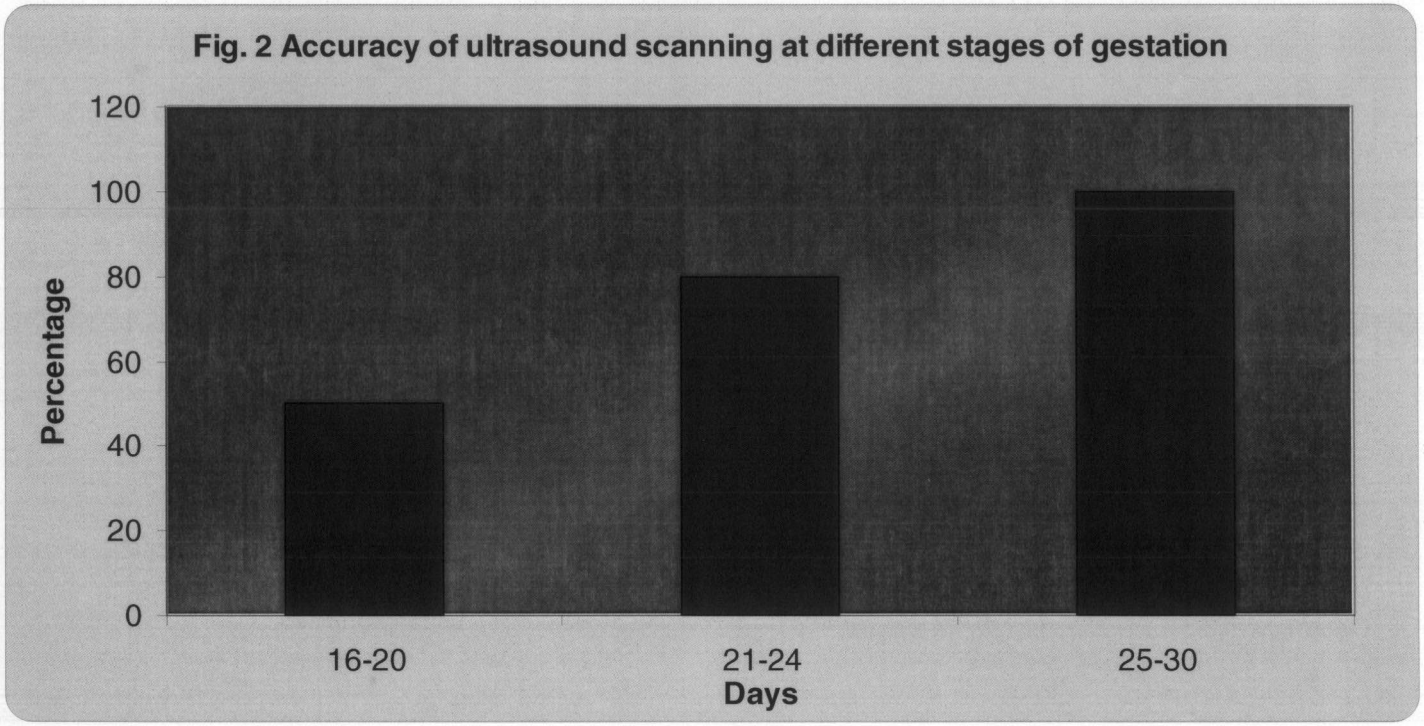


Fig. 2 Accuracy of ultrasound scanning at different stages of gestation
120
100
80
60
40
20
0
Percentage
16-20
21-24
25-30
Days

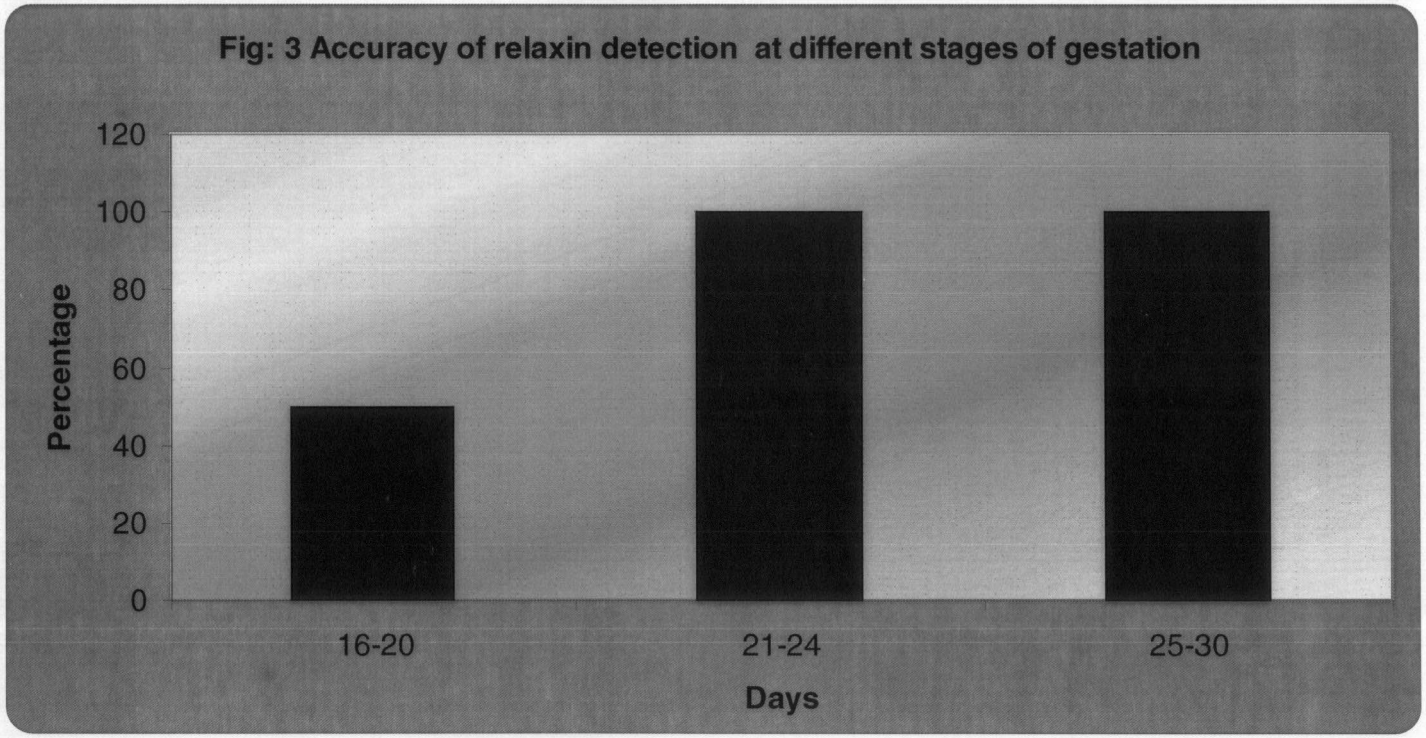


Fig: 3 Accuracy of relaxin detection at different stages of gestation
120
100
80
60
40
20
0
Percentage
16-20
21-24
25-30
Days

Table 3 Haemogram of an mals prior to breedin̲ ar d at d te e
̲estat onal a̲e

Days	Haemoglobin (g/dl)	Packed cell volume (%)	Fry throcyte sedimentation rate (mm/hr
Day Zero	11 56 0 ?7	34 66 0 9	4 6
16 to 20	10 88 0 31	30 77 0 94	14 ɔ 1 (9
21 to ?4	10 24+0 ??	28 ?? 1 0?	17 8 1 8
25 to 30	8 77 0 25	26 0 94	1 76 1 4

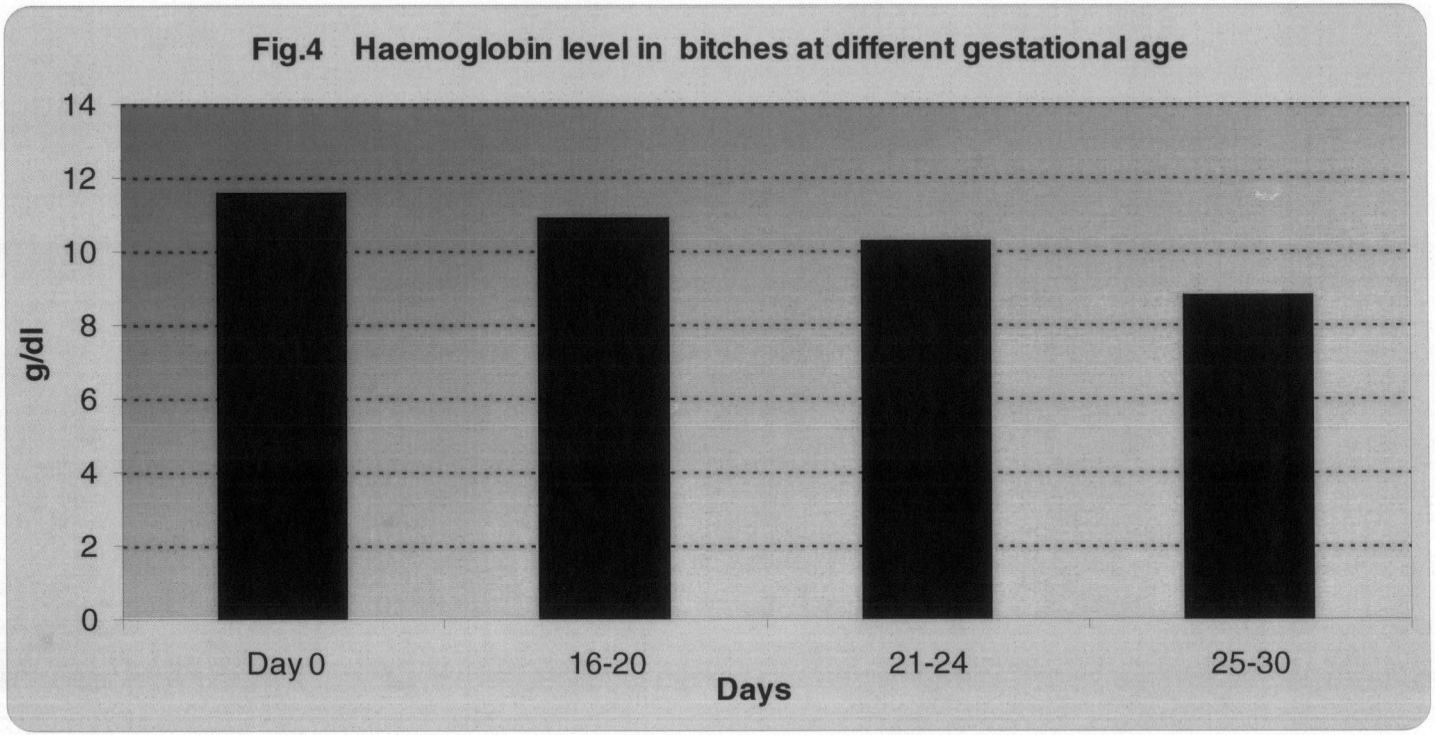


Fig.4 Haemoglobin level in bitches at different gestational age
14
12
10
8
6
4
2
0
g/dl
Day 0
16-20
21-24
25-30
Days

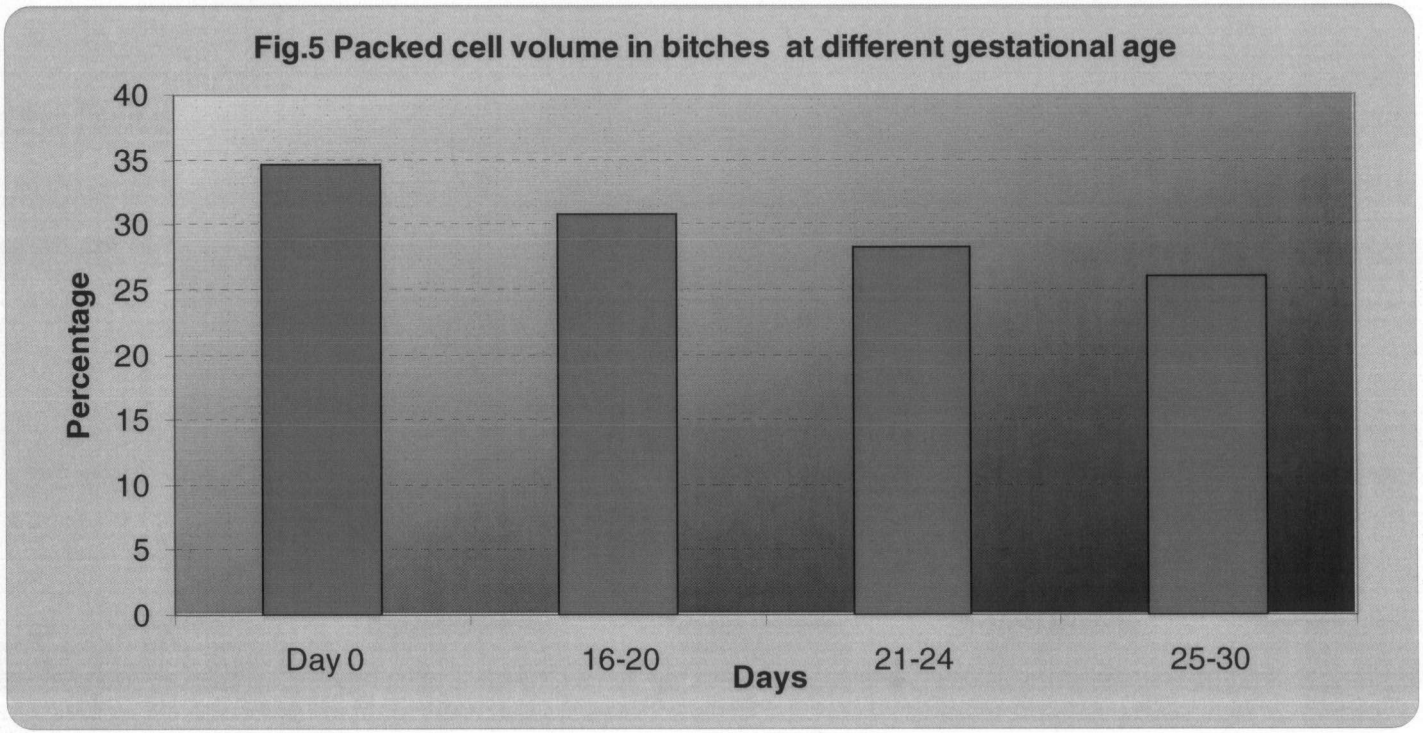


Fig.5 Packed cell volume in bitches at different gestational age
Percentage
40
35
30
25
20
15
10
5
0
Day 0
16-20
21-24
25-30
Days

4 4 3 Erythrocyte Sedimentation Rate (ESR)

Erythrocyte sed nen a on ate a da 0 16 to 0 1 t 4 nd da of gestat on ere fo nd to be 4 6 0 33 4 3 1 0) 7 8 1 8 ?1 76 1 47 ı m h resp ct vely (Table 3 and F 6) ESR v n r ase as pregnancy advanced Stat st cal nal s e eal d s n f a a bet een the val es at d fferent gestat onal age (P 0 01)

4 5 BODY WEIGHT

Body we ght vas recorded n ten an n al a day 0 6 (4 ? 0 days respect ely It as fo nd tl at the bod e gh r d and recorded as ? 83 1 98 ?6 6 93 7 78 1 86 a d 9 (9 4 d ff rent per od of gestat on (Table 4 and F ı 7) It as obs r d e ght gradually ncrea ed fro n da 0 to day 30 (P 0 0)

4 6 GESTATIONAL ACCIDLNTS

In the presen study three cases of abort on o ıe cas f p fou pseudopregnanc es were recorded The n de ce o p d p ab rt on and py n et a as 1 1 4 8 ɔ7 and 86 p r en e ı c f 8 Pla es ?3 a d ?4)

4 7 GESTATION LI NG T H

In the present st dy gestat on le gtl a ged bet e 9 o 68 1 an a erage of 6 38 days

4 8 LITTER SI/F

Var ed bet een hree to n ne p ps w th an averaɡe f 7 p p (Pla e 2ɔ)

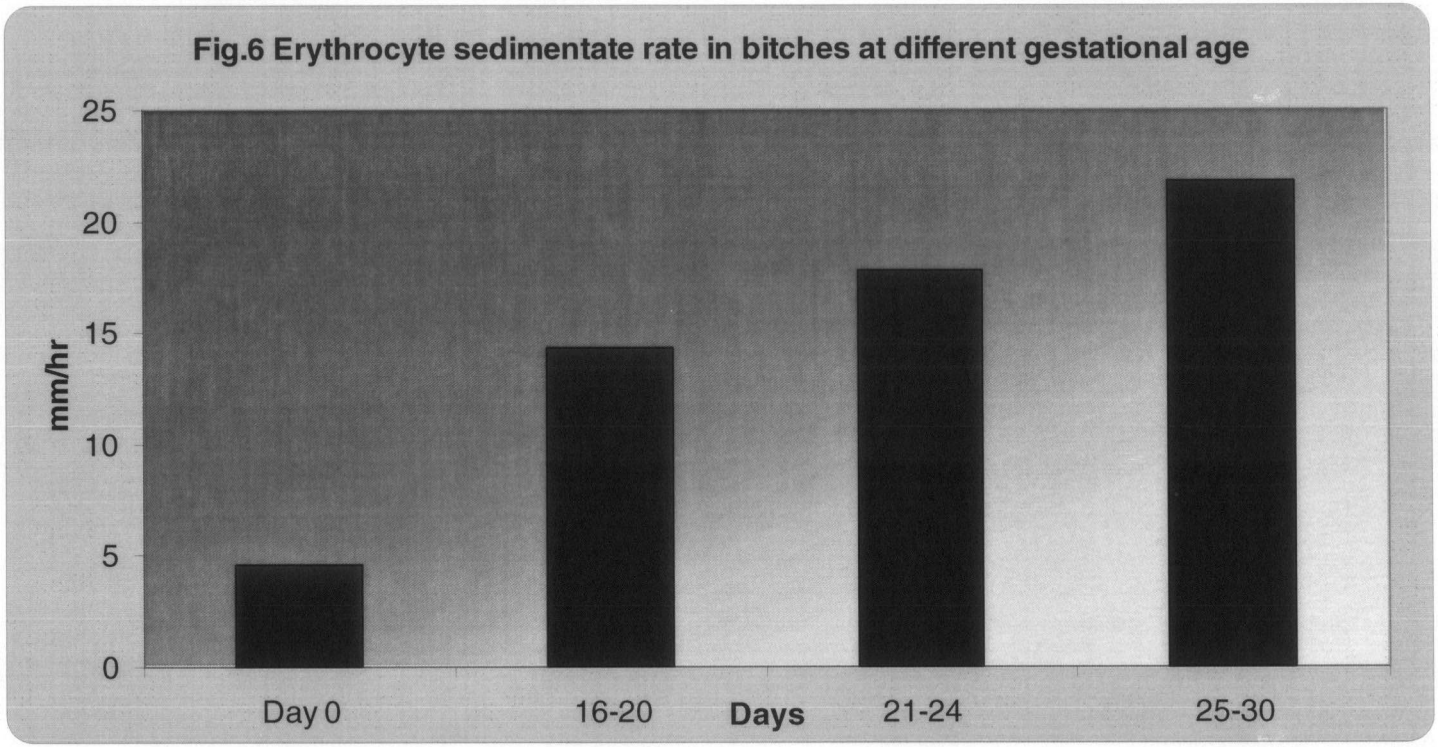


Fig.6 Erythrocyte sedimentate rate in bitches at different gestational age
25
20
15
10
5
0
mm/hr
Day 0
16-20
Days
21-24
25-30

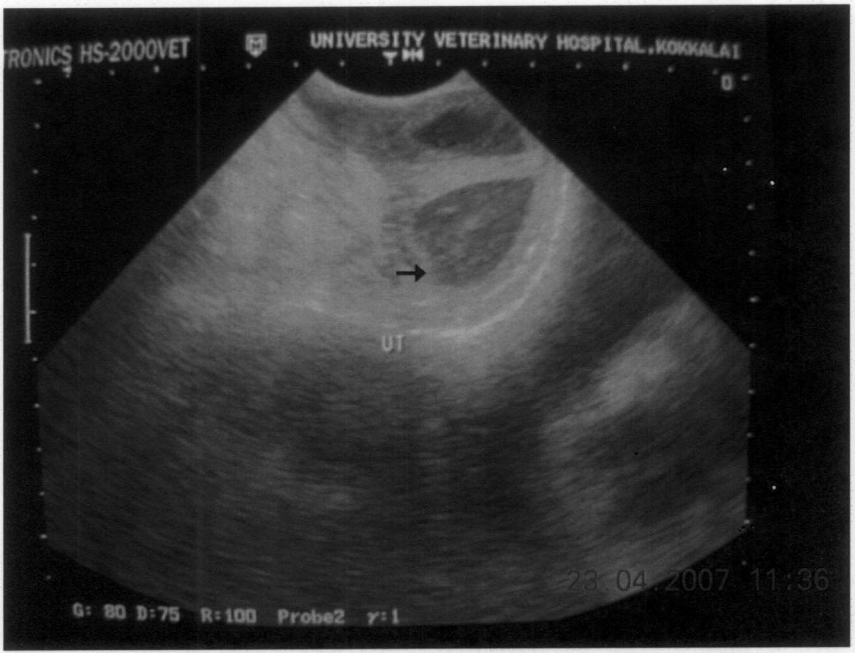


Plate : 23 Sonogram of a uterus with Pyometra.The lumen of the uterus is serpentine and filled with anechoic fluid. There is no evidence of foetal structure.

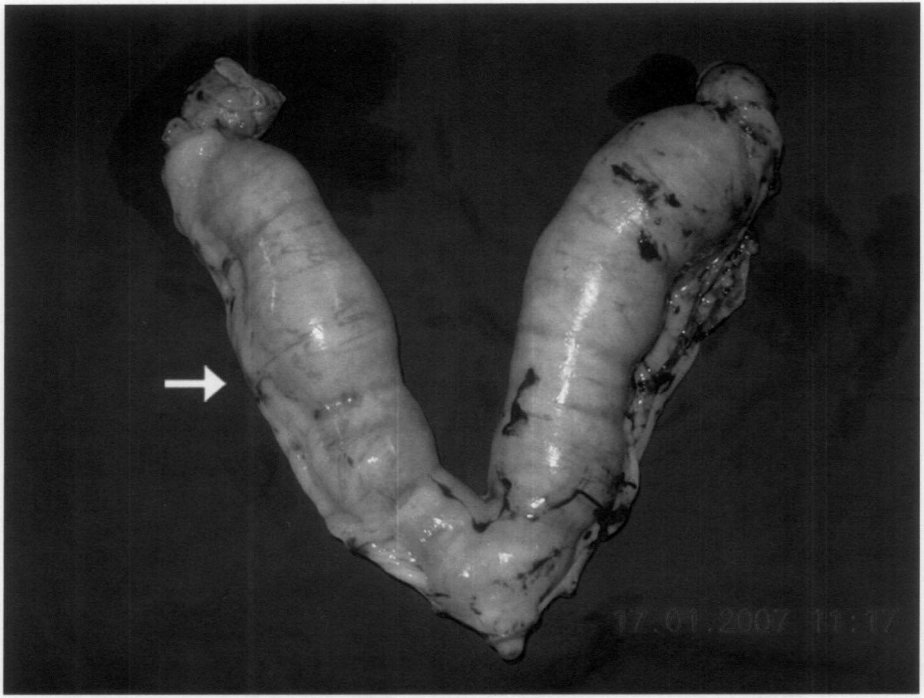


Plate: 24 Excised genetalia from a bitch with pyometra. Note the distended feeling of utreine horns with pus.

Plate : 25 A Labrador bitch with 5 pups.

Table 4 Comparison of body weight of animals prior to breed and a different gestational age

Days	Body Weight (Kg) (n 10)
Day 0	5 8 1 98
16 to 20	26 6 1 9
21 to 24	27 78_1 8(
25 to 30	29 69_1 45

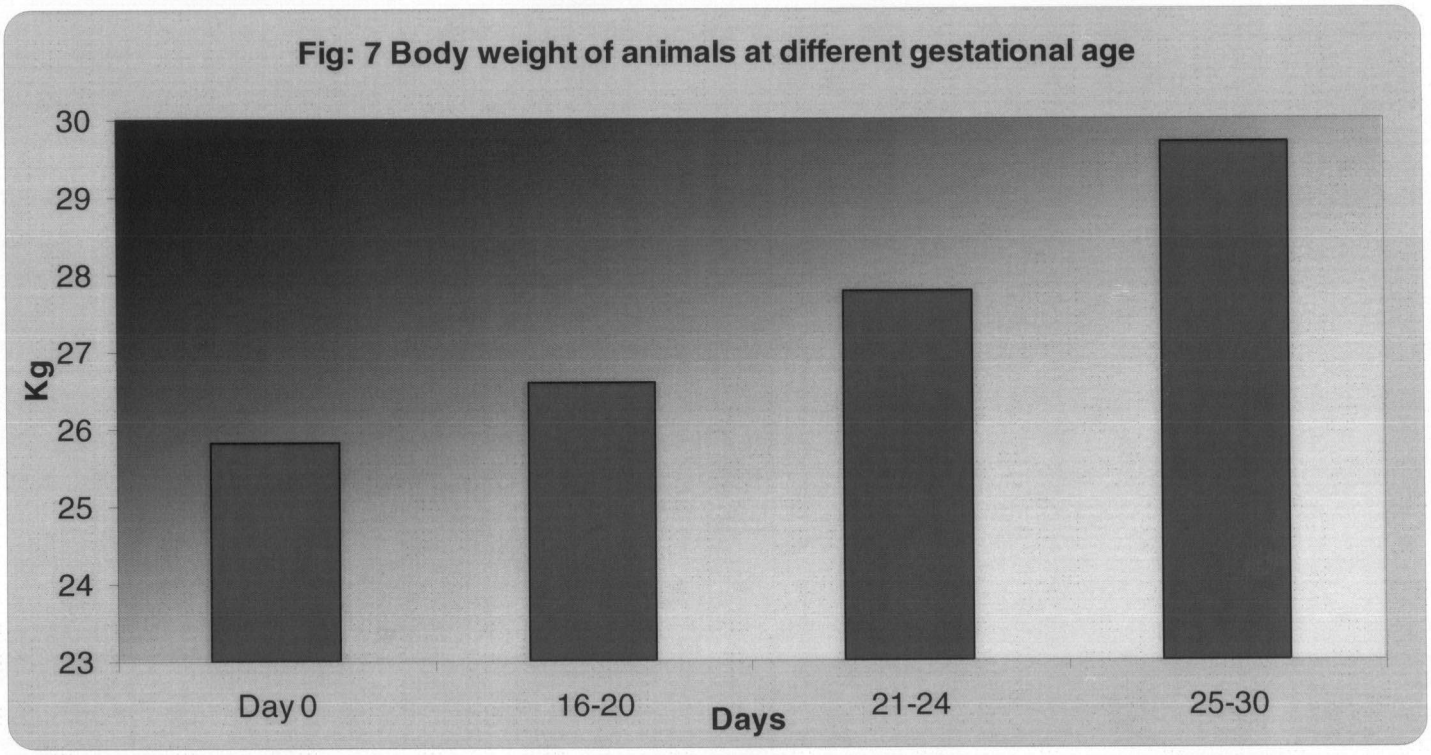
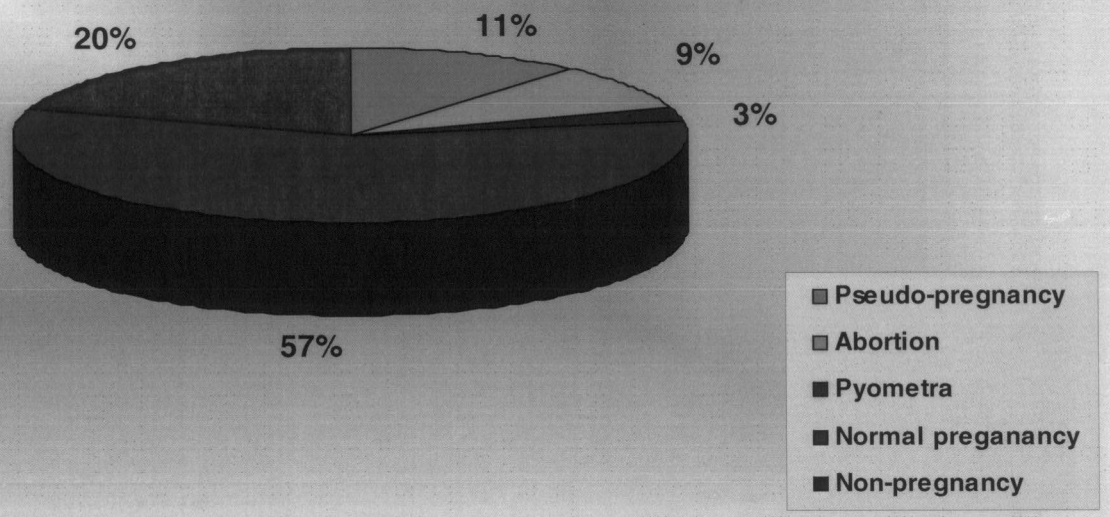


Fig: 7 Body weight of animals at different gestational age
30
29
28
27
26
25
24
23
Kg
Day 0
16-20
21-24
25-30
Days

Fig: 8 Percentage occurrence of various reproductive problems encountered during the course of study



Discussion

5 DISCUSSION

Early diagnosis of pregnancy is essential for commercial success of any dog breeding programme Because it not only reduces the chance of accidental mishap but also offers an opportunity for better management and nutrition to the pregnant bitches Further the knowledge of the dimensions of the genitalia in bitches is important in diagnosis of reproductive disorders particularly those which simulate pregnancy Even though a variety of techniques are now available that can be employed as tools for pregnancy diagnosis the development of an economic quick simple and accurate method for diagnosing pregnancy at an early stage in bitches will be useful for better breeding management and in this context the present study was undertaken to compare the efficacy of abdominal palpation ultrasonography serum relaxin detection for the diagnosis of pregnancy litter size and patholog cal conditions of uterus were carried out and the results were discussed with the available literature

5 1 OPTIMAL TIME FOR BREEDING

Optimal breeding time was advised based on clinico gynaecological examination and exfoliative vaginal cytology (EVC) In the present study more number of animals conce ved as against those bred based on oestrus signs alone (Table 1) This support the view that clinico gynaecological examination and exfol ative vaginal cytology will be useful to find the optimal breeding time in bitches Sim lar observations were reported earlier by Simon and Athman (1998) Becha (2000) and Asha (2005)

5 2 PREGNANCY DIAGNOSIS AT DIFFERENT GESTATIONAL AGE

5 2 1 At 16 to 20 Days Post Breeding (n=10)

Palpation of the abdomen was found to be difficult at 16 to 20 days post breeding to differentiate a pregnant uterus from a non pregnant one Moreover

palpation was hindered by tenseness of abdominal muscles and excessive fat over the abdominal region Results of palpation varied between the animals and varied between the breeds It was found that the palpation was easier in German shepherd bitches compared to Labradors and Rottweilers This is due to their lean body conformation Some animals were very apprehensive and nervous and this made it too difficult to do the palpation in them Similar opinions were given by Sokolowski (1980) Arthur *et al* (1996) England (1998) Thou (1999) and Asha (2005) who opined that pregnancy diagnosis was difficult in obese and nervous bitches

In the present study the percentage accuracy obtained at the gestational age of 16 to 20 days was only 20 percent More negative cases were recorded at this stage because of non appreciable increase in the size of the uterus Pregnancy diagnosis at this stage by trans abdominal palpation was very difficult as distinct uterine swellings could not be appreciated from the abdominal viscera in bitches This is in agreement with the findings of Cartee and Rowles (1984) Shille and Gontarek (1985) Thou (1999) and Asha (2005)

By ultra sound scanning at 16 to 20 days post breeding embryonic vesicle alone could be observed The foetus as well as foetal structure remained undetectable at this stage Accuracy at this stage was 50 percent This finding is in agreement Feldman and Nelson (1996) Kuztritz (2003) and Alacam *et al* (2005)

When serum relaxin detection by rapid immuno migration technique was done at 16 to 20 days 50 per cent of animals gave positive result The earliest positive result obtained was at 20^{th} day post breeding This is in agreement with Arthur *et al* (1996) Concannon (2000) and Kustritz (2003)

5 2 2 At 21 to 24 Days Post Breeding (n 10)

By abdominal palpation percentage of animals found positive at 21 to 24 days of pregnancy was 50 percent Similar findings were reported by Gradil *et al*

(2000) Purswell (2001) Deka *et al* (2004) and Arunmozhi (2005) However Thou (1999) reported more doubtful cases between 20 to 25 days of gestat on among large breeds

When ultrasound scanning was done 21 to 24 days 80 per cent of animals were found to be pregnant by observing the gestational sac with black colour and anechoic texture (Fig 3) Hypo echoic embryo could be observed at this stage Percentage accuracy was 80 at 21 to 24 days of gestation This is in agreement with the findings of Kahn (1994) Cartee and Rowles (1984) From 23 to 25 days foetal heart beat was observed which is in agreement with Feldman and Nelson (1996) and Yeager *et al* (1992) However positive cases obtained in this study were lesser than those reported by Deka *et al* (2004)

When serum relaxin detection test was done at 21 to 24 days all the ten animals gave a positive result suggestive of pregnancy and given an accuracy of 100 per cent This is in agreement with Feldman and Nelson (1996) England (1998) Johnston *et al* (2001) Alacam *et al* (2005) and Ozyurtlu (2006)

5 2 3 At 25 to 30 Days Post Breeding

When abdominal palpation was done in between 25 to 30 days of gestation in 70 per cent of animals foetus could be felt as distinct uterine swelling which is in agreement with the findings of Sokolowski (1985) Yeager and Concannon (1990) and Concannon (2000)

Ultrasound scanning when done at 25 to 30 days of gestation an accuracy of 100 per cent was obtained by monitoring the foetal placenta heart beat and foetal movements Here the uterus had an anechoic lumen with hyper echoic embryo This is in agreement with the findings of Shille and Gontarek (1985) Yeager *et al* (1992) Barr (1998) and Bhadwal and Mirakhur (2000)

When serum relaxm detection was done at 25 to 30 days all the ten animals gave positive result suggestive of pregnancy and given an accuracy of

100 per cent This is in agreement with the findings of Feldman and Nelson (1996) England (1998) Johnston *et al* (2001) Alacam *et al* (2005) and Ozyurtlu (2006)

5 3 PERCENTAGE ACCURACY OF VARIOUS METHODS OF PREGNANCY DIAGNOSIS

5 3 1 Transabdominal Palpation

In the present study pregnancy could not be established at 16 to 20 days When palpation was done in between 21 to 24 and 25 to 30 days post breeding the accuracy obtained was 50% and 70% respectively This study suggests that trans abdom nal palpation was not useful in diagnosing early pregnancy

5 3 2 Transabdominal Ultrasonography

By ultrasound scanning the percentage accuracy at 16 to 20 days was 50% which improved to 80 percent and 100 percent at 21 24 and 25 30 days post breeding respectively Foetal heartbeat could be observed in all the positive cases from 24 days of gestation

5 3 3 Serum Relaxin Detection

The earliest positive result obtained for serum relaxin detect on was obtained at 20th day post breeding and the percentage accuracy was 50% at th s period as against 100% at 21 30 days of gestation Serum relaxin detection was found to be negative in all the three pseudo pregnant cases examined during the course of study This finding is in agreement with Concannon (2000) and Ozyurtlu (2006) Thus it is evident that serum relaxin can be used as a test for differentiating pregnancy from pseudo pregnancy

5 4 HAEMATOLOGICAL STUDIES

5 4 1 Haemoglobin

In this study the haemoglobin concentration in pregnant was lower than the value obtained on the day of breeding From the mean control value of 11 56 +0 27g/dl it was reduced to 10 88+0 25 10 24±0 22 and 8 77±0 22 g/dl at 16 to 20 21 to 24 and 25 to 30 days respectively The lower values obtained at different stages of gestation was due to haemodilution and increased plasma volume (Sastry 1989) Thou (1999) observed a significant decrease n haemoglobin concentration from non pregnant bitches as 14 19+0 2 g/dl to 11 0±0 18 g/dl as pregnancy advanced Similar observations were reported by Asha (2005) who found a significant decrease in haemoglobin concentration from 12 37±0 28 in non pregnant dogs to 10 69+0 2g/dl as pregnancy advanced

5 4 2 Packed Cell Volume

The packed cell volume values obtained before conception and after conception varied significantly (P<0 01) The packed cell volume values of control was 34 66±0 9 per cent where it was reduced to 30 77±1 02 28 22±0 94 26 0+0 94 per cent at 16 to 20 21 to 24 and 25 to 30 days post breeding respectively Thou (1999) also found a decrease in packed cell volume as pregnancy progressed and found the mean values on days 21 to 25 30 to 35 and 45 to 50 in pregnant and non pregnant dogs as 46 33+0 85 43 33+0 58 and 38 67+0 53 per cent respectively Asha (2005) also reported a decrease in PCV with increase in ESR in pregnancy and concluded that the control value was 45 0+1 15 percentage where it decreased to 36 88+0 8 percentage in late gestation

5 4 3 Erythrocyte Sedimentation Rate

The ESR rate increased in animals after conception when compared to the value obtained at the day of breeding The rapid increase in ESR in pregnancy s

favoured by elevated levels of fibrinogen (Henry 1996) ESR increased from control value of 4 6±0 33 to 14 3+1 2 17 8±1 4 and 21 76+1 4 mm/hr respectively at 16 to 20 21 to 24 and 25 to 30 days respectively Thou (1999) found an ESR in pregnant bitches ranging between 8 5 to 19 33 mm/hr and varied significantly compared to non pregnant animals ranging between 5 62 to 5 88 mm/hr

5 5 BODY WEIGHT

In the present study there was significant variation in body weight ($P<0.05$) before and after conception The present study revealed a slow and steady increase in the body weight of animals as the pregnancy progressed The control value of body weight before conception was 25 83+1 8Kg which increased to 26 6+1 9 26 78+1 8 and 29 6±1 8 at 16 to 20 21 to 24 and 25 to 30 days of gestation respectively Similar findings were given by Thou (1999) and Arthur *et al* (2001)

5 6 GESTATIONAL ACCIDENTS

Here the percentage of pseudo pregnancy gave a figure of 11 per cent and abortion was 9 per cent (Fig 8) Similar findings were reported by Ettinger and Feldman (2000) and Bhadwal and Mirakhur (2001)

5 7 GESTATION LENGTGH

In the present study gestation length varied from 59 to 68 days in large dogs with an average of 63 38 days Similar findings were reported by Sokolowski (1980) Concannon (2000) and Asha (2005) According to them the gestation length in bitches varied from 58 to 65 days

5 8 LITTER SIZE

The litter size varied between 3 to 9 pups with an average of 5 7 pups per bitch This is in agreement with Concannon (1986) and Asha (2005)

CONCLUSION

It was found that trans abdominal palpation was unreliable during early pregnancy diagnosis Many of the dogs resisted palpation during early days of gestation and hence this method cannot be employed under field conditions

Ultrasound scanning was accurate and reliable for early pregnancy diagnosis as it gave a figure of 80 per cent accuracy when performed in between 21 to 24 days of gestation However the limitation is that only skilled persons can perform ultrasonography and exact interpretation of the results need high technical knowledge

Among all the methods serum relaxin detection was the most easiest and reliable method for early pregnancy diagnosis as it could confirm pregnancy by 20^{th} day post breeding The major advantage of serum relaxin detection is that pseudo pregnancy pyometra and other uterine pathological conditions would not interfere with the result Moreover it could be done easily under field conditions as an in house assay even by a dog owner

Summary

6 SUMMARY

The current study entitled Detection of serum relaxin as a diagnostic tool for early pregnancy diagnosis in bitches was undertaken to compare different methods of pregnancy diagnosis like transabdominal palpation ultrasound scanning and relaxin detection at different stages of gestation

Animals for the study comprised of 45 apparently healthy bitches which was brought for exfoliative vag nal cytology for finding the opt mal breeding t me Out of this ten animals were selected at random and subjected to different methods of pregnancy diagnosis Body weights of animals were recorded prior to breeding and at different gestational age

Conception rate was more in those animals which were bred based on climco gynaecological examination and exfoliative vaginal cytology than those bred based upon oestrous signs alone This supports the view that climco gynaecological examination and exfoliative vaginal cytology will enhance the conception rate in bitches

In the present study conducted in ten animals it was found that transabdominal palpation was very difficult to diagnose pregnancy when performed in between 16 to 20 days post breeding This is because of non appreciable increase in the size of the uterus The accuracy obtained at this stage was only 20 percent When palpat on was done at 21 to 24 days of gestation uterine swellings could be felt more easily when compared to the early gestation and the accuracy obtained was 50 percent The accuracy obtained at 25 to 30 days of gestation was 70 percent It was observed that abdominal palpation was difficult to perform in obese and nervous bitches

By ultrasound scanning at 16 to 20 days embryonic vesicle was visualized in 50 percent of the cases At 21 to 24 days gestational sacs conta n ng foetal tissue suspended in amniot c fluid could be seen The percentage accuracy

at this period was 80 percent All ten animals were found to be positive by observing foetus as well as foetal heart beat by 25 to 30 days of gestation The advantage of ultrasonography over abdominal palpation was it was more accurate in diagnosing pregnancy from pseudo pregnancy after 25 days of gestation

All the ten animals were subjected to serum relaxin detection test and t was found that serum relaxin could identify pregnancy by 20 days of gestation and the percentage accuracy was 50 percent when done between 16 to 20 days post breeding The earliest result obtained was on 20th day post breeding This figure improved to 100 percent at 21 to 24 and 25 to 30 days of gestation This shows that serum relaxin detection is a defin te tool for early pregnancy diagnosis in bitches

There was significant variation in body weight of animals as the pregnancy progressed Body weight of animals steadily increased from day of breeding to various stages of gestation ($P<0$ 01)

Likewise significant variation was observed in the haematological profile The haemoglobin and packed cell volume decreased as the pregnancy progressed but the erythrocyte sedimentation rate showed a rap d increase when compared to the value obtained at the day of breeding ($P<0$ 01)

From the present study it could be concluded that abdominal palpation could not be relied for early pregnancy diagnosis But ultrasound scanning could be used as a more accurate method for diagnosing pregnancy after 24 days post breeding Serum relaxin detection will serve as a better tool in early pregnancy d agnosis before day 20 The advantage is that the result for serum relaxin detection was not influenced by pseudo pregnancy or pyometra and the test can be performed even by the dog owner as an in house assay Hence it is recommended that serum relaxin detect on is quick simple easy to perform and accurate in diagnosing early pregnancy in bitch

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DETECTION OF SERUM RELAXIN AS A DIAGNOSTIC TOOL FOR EARLY PREGNANCY DIAGNOSIS IN BITCHES

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ABSTRACT

With the object of finding a suitable and reliable method of early pregnancy diagnosis in bitches the study was undertaken to investigate the efficacy of trans abdominal palpation ultrasound scanning and relaxin detection was conducted

The study consisted of 45 apparently healthy bitches which were brought to the clinics for finding the optimal breeding time Out of this ten animals were selected at random for pregnancy diagnosis and were subjected to different methods of pregnancy diagnosis at different gestational age 16 to 20 days 21 to 24 days and 25 to 30 days post breeding Blood samples were collected for the estimation of haemoglobin packed cell volume and erythrocyte sedimentation rate at the day of breeding and also at the above gestation periods Body weights were recorded at the day of breeding and also at different gestation periods

In the present study it was found that abdominal palpation was difficult in diagnosing pregnancy between 16 to 20 days of gestation When palpation was done in between 21 to 24 and 25 to 30 days post breeding the accuracy obtained was 50% and 70% respectively This study suggests that trans abdominal palpation was not useful in diagnosing early pregnancy

By ultrasound scanning the percentage accuracy at 16 to 20 days was 50% which improved to 80 percent and 100 percent at 21 24 and 25 30 days post breeding respectively Foetal heartbeat could be observed in all the positive cases from 24 days of gestation Pseudo pregnancy pyometra and abortion could be easily identified by this method

The earliest positive result obtained for serum relaxin detection was obta ned at 20th day post breeding and the percentage accuracy was 50% at this period as against 100% at 21 30 days of gestation In the present study it was

found that serum relaxin test was not influenced by pseudo-pregnancy and uterine pathological conditions like pyometra.

There was significant variation in haemogram ($P < 0.01$) at the day of breeding and at different gestational age. Haemoglobin concentration at 16-20, 21-24 and 25-30 days of gestation were 10.88 ± 0.31, 10.24 ± 0.22, 8.77 ± 0.28g/dl, which was lower than the value 11.56 ± 0.27 obtained prior to breeding.

The packed cell volume values were 34.66 ± 0.9, 30.77 ± 0.94, 28.22 ± 1.02 and 26 ± 0.94 percent at day 0, 16-20, 21-24, 25-30 days post breeding. There was significant variation in the values before and after conception.

There was significant variation in erythrocyte sedimentation rate between day zero and at different gestational age. The values obtained varied significantly and recorded as 4.6 ± 0.33, 14.3 ± 1.09, 17.8 ± 1.28 and 21.76 ± 1.47mm/hr at day 0, 16-20, 21-24 and 25-30 days of gestation respectively.

The body weight of all the ten animals varied significantly ($P<0.01$). It was observed that the body weight had shown a steady and progressive increase as the pregnancy advanced.

The study revealed that abdominal palpation was not very useful in diagnosing early pregnancy. By ultrasound scanning, uterus as well as foetus could be visualized after 23 days of gestation. Serum relaxin detection could be used as an early tool for pregnancy diagnosis in bitches from 20 days post breeding. Results of the present study suggest that the relaxin test was accurate in diagnosing early pregnancy and its advantage being that it could be conducted and interpreted easily by a dog breeder or a dog owner. It could be concluded that detection of serum relaxin is a quick, simple and accurate tool for diagnosing early pregnancy under field conditions.