CHAPTER III

3.0 MATERIALS AND METHODS

3.1 Location of study

This study was undertaken at three private farms in Gua Musang and Kuala Krai, Kelantan. These farms are under the Department of Veterinary Service programs for Targeted Area Concentration (TAC) Chiku 3 (Figure 3.1). Data collection started from January 2006 until December 2007. During this study, daily temperature ranged from 24^{o}C to 32^{o}C and relative humidity was 80 % with intermittent rain throughout the year. The wet season is the east-coast monsoon season from November until January.

3.2 Experimental animals

The animals involved in the study aged between 3 to 7 years old and mainly of the Charolais crossbreds (Plate 3.1), Brahman crossbreds (Plate 3.2) and Kedah-Kelantan (indigenous cattle of Malaysia) breed (Plate 3.3). The numbers of cows selected for this study were 41 from Farm 1, 42 from Farm 2, and 19 from Farm 3, which in total involved 102 cows selected for this study. All cows were examined rectally for pregnancy diagnosis. Non-pregnant cows were selected for the controlled internal drug release (CIDR) and artificial insemination (AI) programme.

3.3 Management system

The animals from the 3 private farms were bred in 3 different management systems which were the intensive (Farm 1), semi-intensive (Farm 2) and integration under oil palm plantation (Farm 3).

3.3.1 Intensive system (Farm 1)

Intensive system is where all the animals are kept in the shed with provided feed. This farm is owned by Tuan Haji Rosdi Bin Abd. Rahman, with an area of 130 acres and located at Telekong village, Kuala Krai,

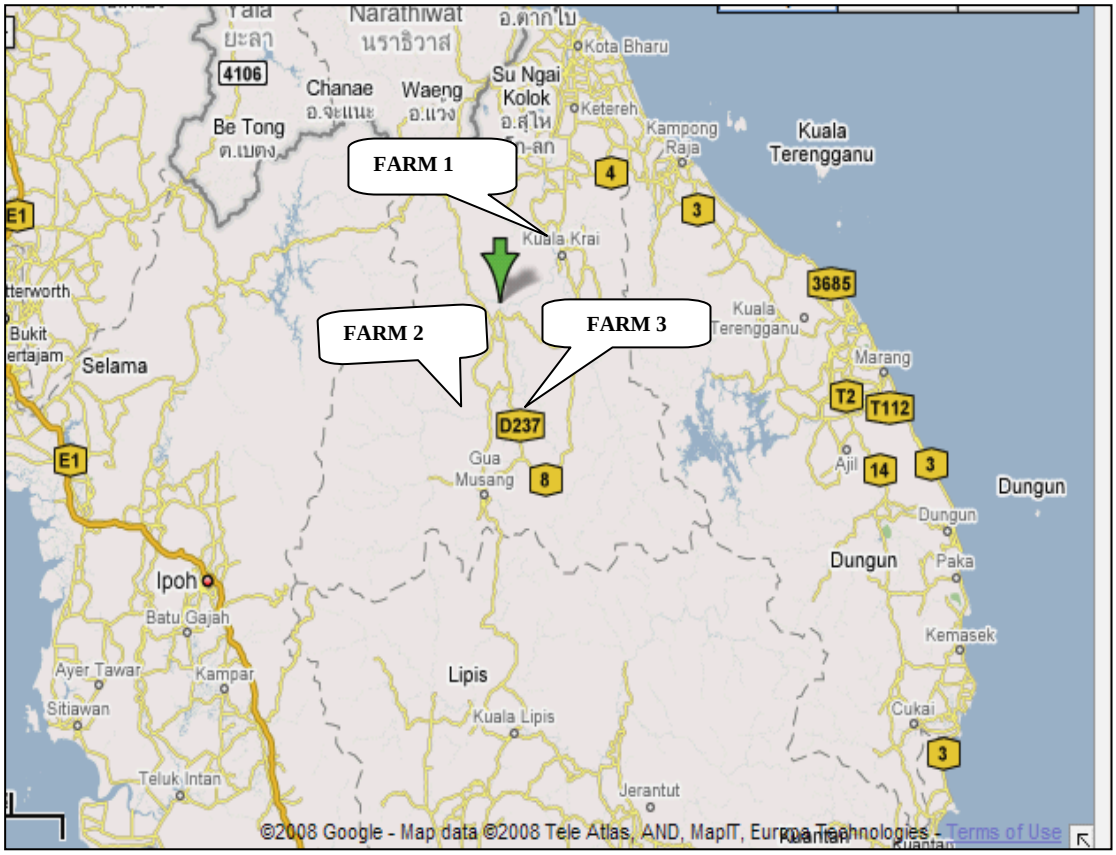

Figure 3.1 Location of farms under study

Plate 3.1 Charolais crossbred cow

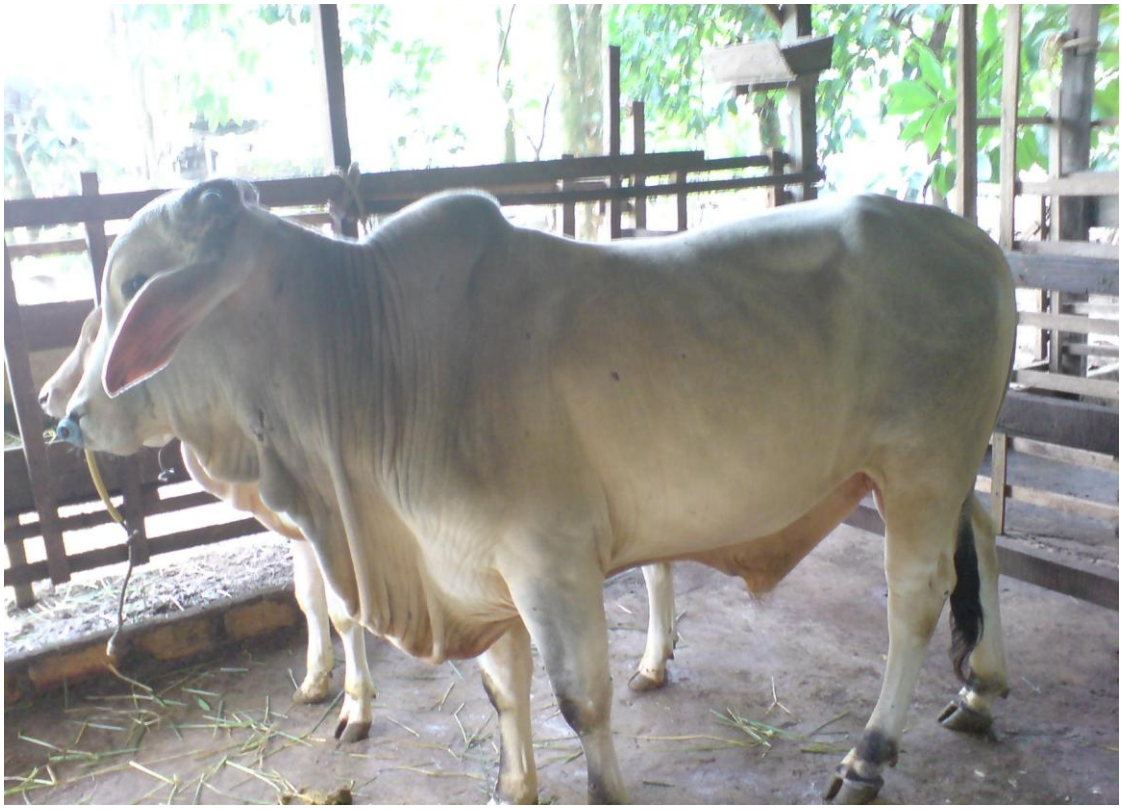

Plate 3.2 Brahman crossbred cow

Plate 3.3 Kedah-Kelantan cow

Kelantan (Plate 3.4). There are more than 300 heads of Charolais crossbreds cows. In this farm cows were fed with cut and carry Napier grass and oil palm leaves twice daily in the morning and evening. Palm Kernel Cake (PKC) and other concentrate pellets were supplemented daily, while drinking water was supplied *ad libitum*.

Plastic ear tags were used for animal identification while regular deworming and annual vaccinations were done once in 6 month by the officers from the Department Veterinary Services, Kelantan.

3.3.2 Semi-intensive system (Farm 2)

Semi-intensive system is where the cattle are allowed to graze freely either in a fenced pasture or in a public pasture for a fixed period of time during the study (Plate 3.5). The cattle are then herded back to the cattle sheds for supplementary feeding and shelter during the night. This farm is owned by a group of farmers who themselves owned 1 to 5 heads of cattle per person. The cattle were allowed to graze twice daily, in

the morning and evening. Occasionally the cows were given Palm Kernel Cake (PKC) and other concentrates. Water and mineral licks were also provided *ad libitum* in the shed. The animals were identified by plastic ear tags while regular deworming and annual vaccination were done once in 6 months by the officers from the Department of Veterinary Services, Kelantan.

3.3.3 Integration system (Farm 3)

Integrated farming system is where the cattle are reared in major plantation crops like oil palm, rubber, coconut and fruit orchards. Normally, these plantations are covered with undergrowth that required chemical control. Therefore, integration with livestock will reduce herbicide usage as well as costs of fertilizers. The integration farm is owned by En. Zulkifli bin Mohd Zain (Plate 3.6). The herd size is more than 400 heads and the cows were allowed to graze under the oil palm plantation. About 60 to 79 plants species are available for ruminant feeding in the oil palm plantations. About 70% of the plants species were reported to be edible and used as forages for rearing cattle in the oil palm plantation (Chen *et al*., 1978; Wan Mohammad, 1978; Hassan and Abdullah, 1991). This farm is situated at the Federal Land Development Authority (FELDA) estate, Aring 6, Gua Musang, Kelantan.

The animals were identified by plastic ear tags while regular deworming and annual vaccination were done once in 6 month by the officers from the Department of Veterinary Services, Kelantan.

Plate 3.4 Intensive system farm (Farm 1)

Plate 3.5 Semi-intensive system farm (Farm 2)

Plate 3.6 Integration under oil palm plantation (Farm 3)

3.4 Synchronization of estrus

Non pregnant cows were selected for the synchronization of estrus, using the Controlled Internal Drug Released (CIDR) implant (Plate 3.7). The procedures for the estrus synchronization are shown in Figure 3.2.

3.4.1 Insertion of controlled internal drug release (CIDR)

Controlled internal drug release (CIDR) implant with attached estradiol benzoate capsule (Plate 3.8) was inserted into the vagina of the cow using the CIDR applicator. Prior to CIDR insertion, the applicator was lubricated with K-Y lubricating jelly. The CIDR applicator was gently inserted into the vagina until it reached about 10 cm - 12 cm in dept. The CIDR implant was then released into vagina. Once the CIDR implant was released, the applicator was withdrawn from the vagina, and the drawstring of the CIDR implant was left hanging outside the vagina. The string was used during

withdrawal of the CIDR implant from the vagina. After the insertion, the applicator was cleaned with potassium permanganate solution and re-used for the next cow.

3.4.2 Removal of CIDR and artificial insemination (AI)

The CIDR was removed from the cows after 7 days of implantation. It was done by pulling drawstring which was left hanging outside the vagina. On day 8, estradiol benzoate (EB) was injected to the cow and twenty four hour after EB injection (Day 9), all the synchronized cows were artificially inseminated with frozen semen provided by the Department of Veterinary Services, Kelantan. Artificial insemination (AI) was done by DVS officers.

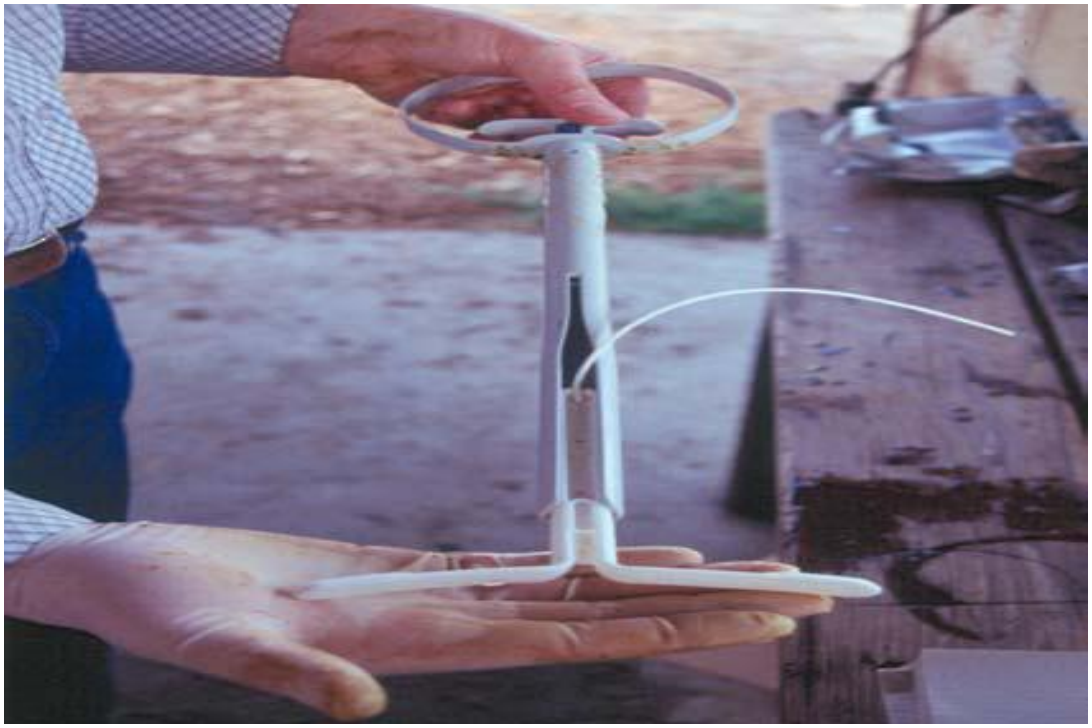

Plate 3.7 CIDR and applicator

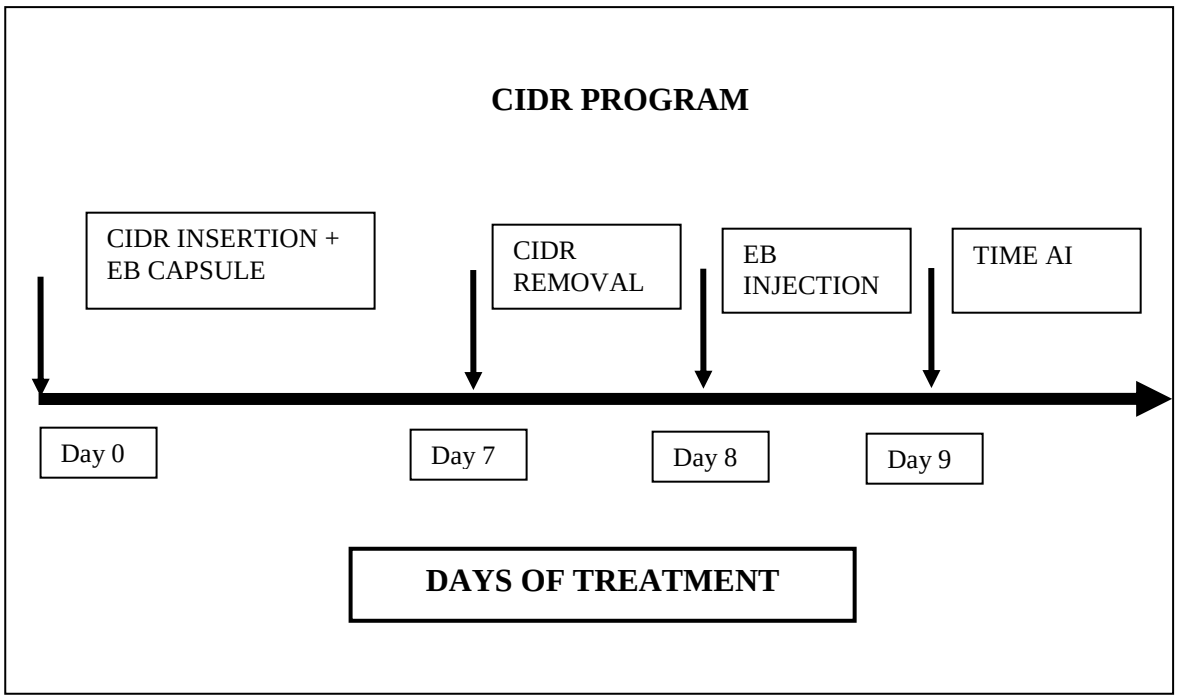

Figure 3.2 The procedures for estrus synchronization in cows

Plate 3.8 Estradiol benzoate solution and estradiol capsule

3.5 Blood Collection for hormone profiles

Blood collections were done only in semi intensive farm with 28 cows (Charolais crossbreds = 8, Brahman crossbreds = 13, KK = 7). Blood was collected from the jugular vein of the cow. The neck of the cow was rubbed and pressed lightly to detect the jugular vein and blood sample was collected using jugular venipuncture 10.0 ml (BD Franklin Lakes NJ USA). Blood samples were collected on day 0 (day of CIDR insertion), 3, 7, 8, 9, 12 and 14 of the experiment (Figure 3.3) to characterize progesterone and estradiol concentrations. All collected blood samples were immediately stored in an ice box and then centrifuged at 3,500 x g for 10 min. Serum was then decanted and stored at -20°C until assayed. Concentrations of progesterone and estradiol in serum were assayed using a Coat-A-Count assay kit (Immunotech A Beckman Coulter Company). Three additional quality control samples were also included using plasma with low, medium, and high concentrations of progesterone and estradiol. Each sample volume was used with samples containing low, medium, or high concentrations of progesterone and estradiol obtained from cattle treated with varying amounts of progesterone and estradiol.

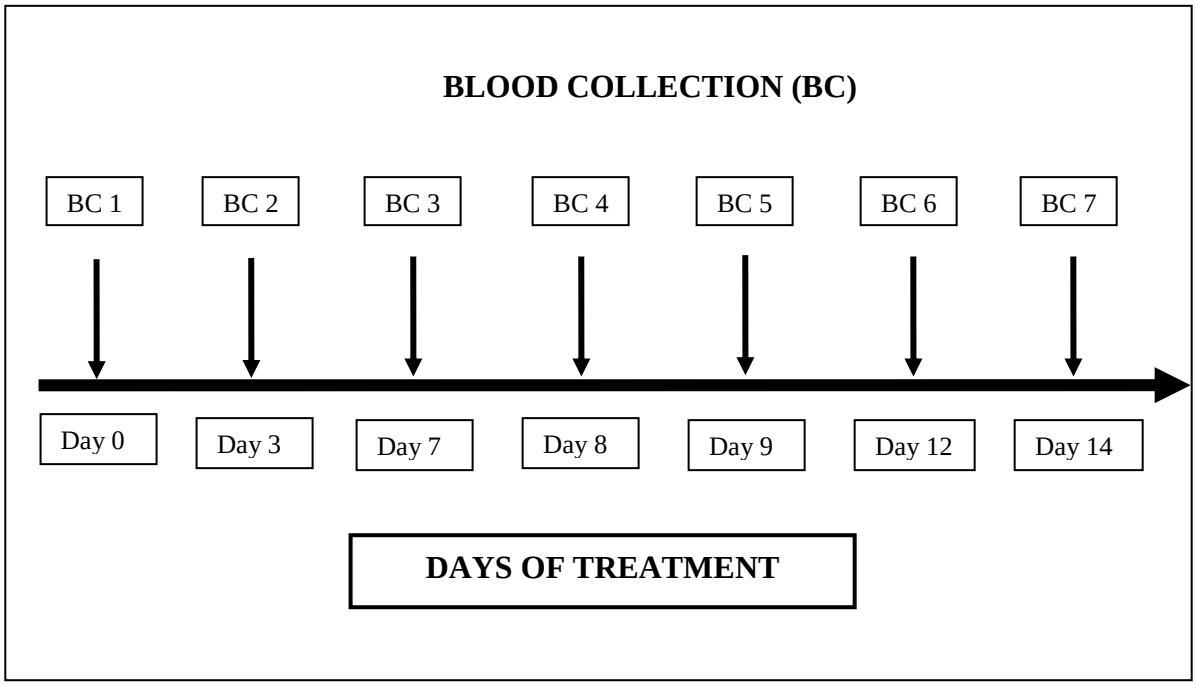

Figure 3.3 The procedure for blood collection (BC) in cows

3.6 Radioimmunoassay (RIA)

Two types of reproductive hormones were analyzed using the radioimmunoassay procedure. These two types of hormone were:

1. Progesterone (P4)
2. Estradiol (E2)

The basic principle of RIA technique was based on the competition between the labelled and unlabelled antigens for the limited binding site on antibodies. The antigens will combine with the antibodies to form the antigen–antibody complex. In this experiment, tracer isotope was used to label the antigens and referred to as the "hot" antigen. The unlabelled antigens were known as the "cold" antigens. Tritium-3 (H-3) was a common isotope used for labelling the steroid hormones such as progesterone and estrogen whereas, radioactive iodine-125 (I^{125}) for labelling the protein hormones such as LH, FSH, TSH, hCG, PRL and GH. However, in this experiment, I^{125} was used to label the entire reproductive hormone (P4, E2 and LH). When the labelled and unlabelled antigens were incubated together with the antibodies, the labelled antigens would compete with unlabelled antigens in the animal's blood samples for a limited binding site on antibodies thus produced the antibody-antigen complex. The reactions occur is shown as:

Ag	+	*Ag	+	Ab	→	AgAb*Ag	+	Ag	+	*Ag
(Unlabelled antigen)		(Labelled antigen)		(Antibody)		(Antigen-antibody complex)		Unlabelled antigen)		(Labelled antigen)

(Yalow, 1980)

For the reactions to reach the equilibrium, the assays were incubated for 3 hours at room temperature (27^oC). It could also be incubated for only one hour but at higher temperature (37^oC) to reach equilibrium. After 3 hours of incubation, there were still unlabelled and labelled antigens remaining in the assay. All the free antigens were discarded, whereby the bound complexes (antigen-antibody complex) remained in the antibody-coated tubes. Then, the tubes were counted using gamma counter to determine

the radioactive in the tubes. The count was inversely related to the amount of unlabelled antigens present in the blood samples. The quantities of unlabelled antigens in the samples were determined by comparing the count to the calibration/standard curve.

The calibration/standard curve was obtained by using known amounts of the same hormone but at several different concentrations (supplied by manufacturer). Using the standard curve, the unknown amount of hormones (in the blood samples) were compared with the amount of radioactive present in the known amount of hormones.

3.6.1 Principle procedure for progesterone and estradiol assay estimation

The principle of assay estimation for progesterone and estradiol was based on the Coat-A-Count's of progesterone and estradiol procedurer. Radioactive Iodine, I^{125} labelled progesterone and estradiol competed with respectively unlabelled antigens for progesterone and estradiol in the serum (blood samples collected from the cows) for the limited antibody binding sites. After three hours of incubation, separation of antigen-antibody complex from the free antigens (the labelled and unlabelled antigens) was achieved by decanting the remaining into the sink. All the free antigens were discarded whereby the bound complexes remained in the antibody-coated tubes. The unlabelled antigens in the tubes were counted by gamma counter.

3.6.2 Radioimmunoassay procedures for progesterone: (Immunotech, 2010)

1. Plain tube: Four plain (uncoated) 12x75 mm polypropylene tubes T (total count) and NSB (nonspecific binding) were labelled in duplicate.

   Coated tube: Fourteen Progesterone Ab-Coated tubes A (Maximum binding) and B through G were labelled in duplicate. Additional antibody-coated tube, were also labelled in duplicate, for control and samples (Plate 3.9).

2. 100 µl of the zero calibrator A were pipetted into the NSB and A tubes, and 100 µl of each of the calibrators B through G into correspondingly labelled tubes (Plate 3.10). 100 µl of each control and sample were pipetted into the prepared tubes (Plate 3.11). They were pipette directly to bottom of the test tubes (Table 3.1).

Table 3.1 Progesterone calibrators

Calibrators	ng/ml	nmol/l
A (MB)	0	0
B	0.1	0.3
C	0.5	1.6
D	2	6.4
E	10	31.8
F	20	63.6
G	40	127.2

3. 1.0 mL of ^{125}I Progesterone were added to each tube (Plate 3.12).
4. The mixture was vortexed for 1 minute.
5. All samples were incubated for 3 hours at room temperature (15-28 ^{o}C)
6. The mixture was decanted thoroughly to remove all visible moisture to enhance precision.
7. All samples were counted in a gamma counter (Plate 3.13).

3.6.3 Radioimmunoassay procedures for estradiol: (Immunotech, 2010)

1. Plain tube: Four plain (uncoated) 12x75 mm polypropylene tubes T (total count) and NSB (nonspecific binding) were labeled in duplicate.

   Coated tube: Fourteen Estradiol Ab-Coated tubes A (Maximum binding) and B through G were labelled in duplicate. Additional antibody-coated tube, were also labelled in duplicate, for control and samples.

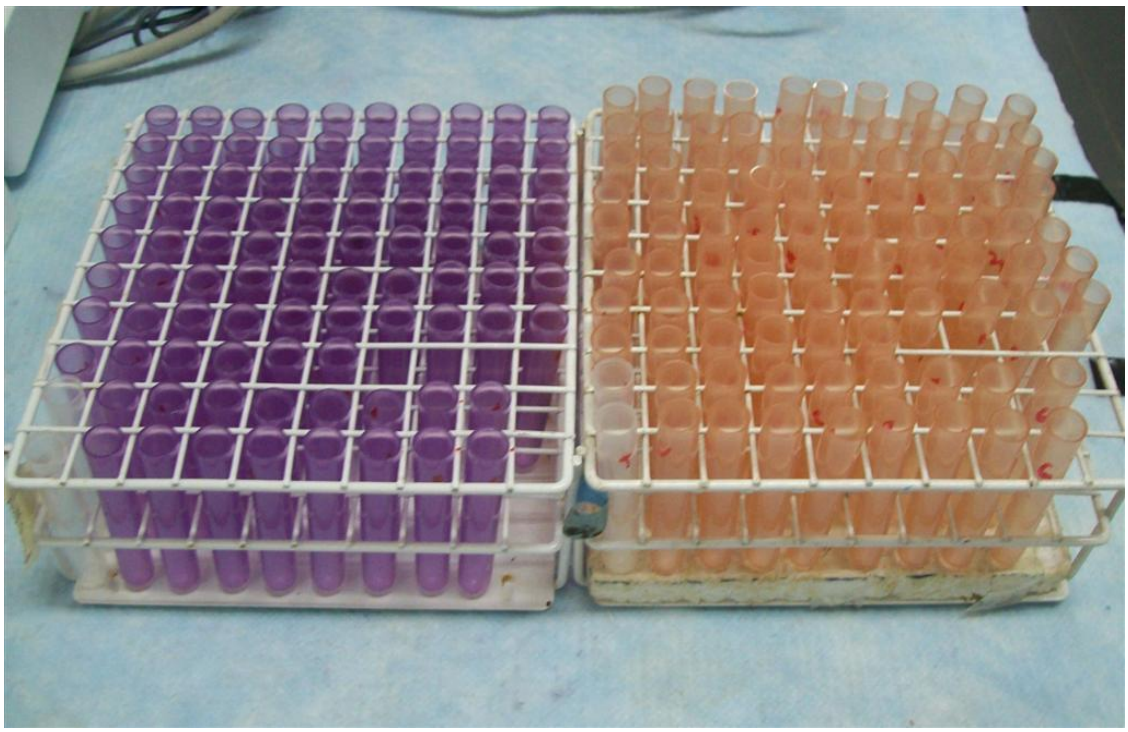

Plate 3.9 Progesterone (orange) and estradiol tubes (purple)

Plate 3.10 Control (pool) serums

L = Low
M = Medium
H = High

Plate 3.11 Progesterone calibrators

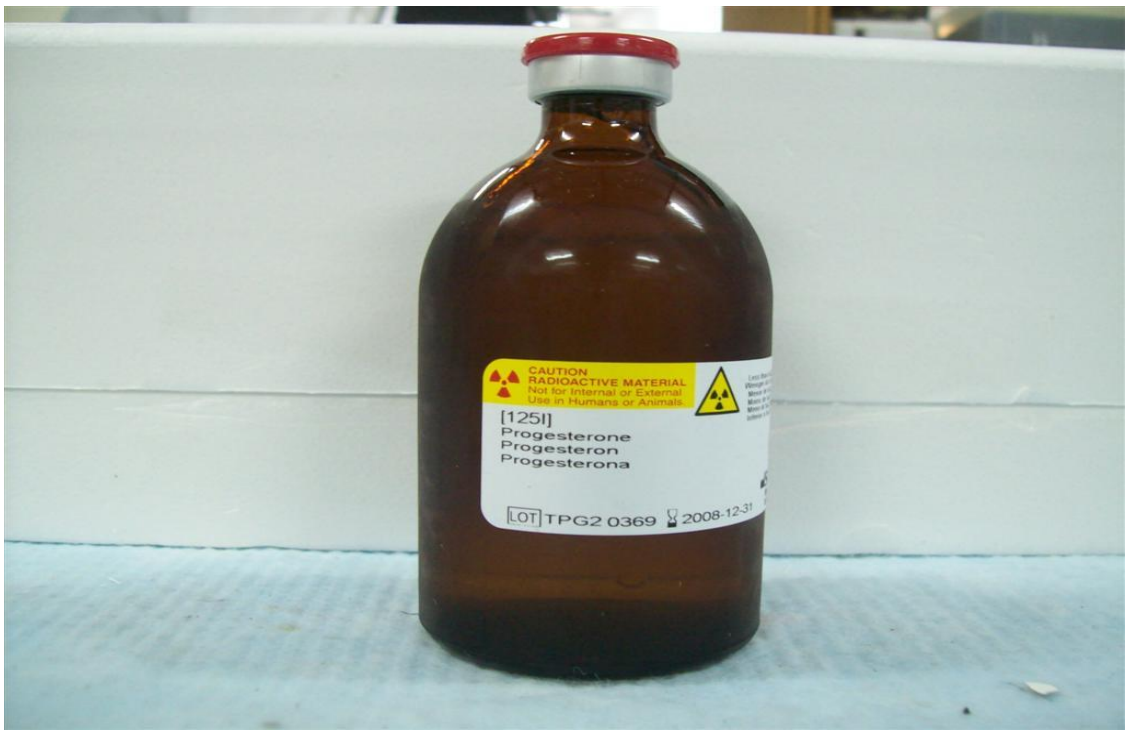

Plate 3.12 Radioactive iodine-125

Plate 3.13 Gamma counter

2. 100 µl of the zero calibrator A were pipettet into the NSB and A tubes, and 100 µl of each of the calibrators B through G into correspondingly labelled tubes. 100 µl of each control and sample were pipetted into the prepared tubes. They were pipetted directly to bottom of the test tubes Table 3.2).

Table 3.2 Estradiol calibrators (Plate 3.14)

Calibrators	pg/ml	pmol/l
A (MB)	0	0
B	20	73
C	50	184
D	150	551
E	500	1836
F	1800	6608
G	3600	13216

3. 1.0 ml of ^{125}I estradiol to were added each tube (Plate 3.14).
4. The mixture was vortexed 1 minute.
5. All samples were incubated for 3 hours at room temperature (15-28 ^{o}C)
6. The mixture was decanted thoroughly to remove all visible moisture to enhance precision.

7. All samples were counted in a gamma counter.

Plate 3.14 Estradiol calibrators

3.7 Pregnancy diagnosis

Pregnancy diagnosis was carried out on the treated cows on day 60 post-insemination using rectal palpation (Plate 3.15). The diagnosis involved retraction and subsequent examination and evaluation of the tubular genitalia and ovaries (Bearden and Fuquay, 2000).

Plate 3.15 Pregnancy diagnosis

3.8 Experimental design

3.8.1 Synchronization of estrus

One hundred and two cows, comprising mainly of Charolais crossbreds (n=52), Brahman crossbreds (n=13) and Kedah-Kelantan (n=37) cows aged between 3 to 7 years were examined rectally to assess ovarian function. Only non pregnant cows with corpus luteum were chosen for the program. Cows with body condition score between 2 to 7 were selected according to scale of 0 to 9 from the most emaciated to the fattest (Richards *et al*., 1986). All cycling cows (n = 102) received CIDR (controlled intravaginal drug release) insertion containing 1.38 g of progesterone (Eazi-Bred™ CIDR®; Pfizer Inc., New Zealand; Day 0) attached with estradiol benzoate (EB) capsule (Pfizer New Zealand Limited) and 2 mg i.m injection of estradiol benzoate (Bomac Laboratories; Day 8) after 24 h of CIDR removal (Based on DVS Kelantan treatment).

Twenty four hours after EB injection (Day 9), all synchronized cows were artificially inseminated with frozen semen provided by the Department of Veterinary Services, Kelantan.

The effect of farming systems and breeds on BCS and fertility responses were observed after the synchronization.

3.8.2 Radioimmunoassay (RIA)

Blood was collected from the jugular vein of the cow. This particular area of the cow was rubbed with hand to detect the jugular vein. Blood samples were then collected by using jugular venipuncture 10.0 ml (BD Franklin Lakes NJ USA) on day 0 (initiation of CIDR insertion), 3, 7, 8, 9, 12 and 14 of the experiment (Plate 3.10). All blood samples were immediately placed in an ice box after collection then centrifuged at 3,500 x g for 10 min. Serum was decanted and stored at -20°C until assayed to determine the concentrations of progesterone and estradiol. Concentrations of progesterone and estradiol in serum were assayed using a Coat-A-Count assay kit (Immunotech A Beckman Coulter Company). Three additional quality control samples were also included using plasma with low, medium, and high concentrations of progesterone and estradiol. Each sample volume was used with samples containing low, medium, or high concentrations of progesterone and estradiol obtained from cattle treated with varying amounts of progesterone and estradiol.

Hormonal level of progesterone and estradiol were analyzed during the treatment.

3.9 Statistical analysis

All data obtained were analyzed using the Statistical Package for Social Science (SPSS) software. Analysis of variance (ANOVA) and Duncan multiple range test were performed to test the significance level ($p<0.05$) on the estrus of the cows with the effect of breed, body condition score, farming system and days of postpartum.