MATERIALS AND METHODS
III. MATERIALS AND METHODS

The data relating to present investigation has been generated from clinical records of pyometra cases in dogs, presented to the Department of Veterinary Gynaecology and Obstetrics, Veterinary College, Hebbal, Bangalore between January 2000 to December 2008 and as well as the data generated from clinical cases of pyometra handled during the course of present investigation, that is between April 2008 to April 2009. A detailed proforma, regarding different parameters considered for the present investigation was specifically prepared and the data was entered for the purpose of analysis.

Clinical findings in pyometra cases of both retrospective and prospective studies were pooled to study the influence of the following factors on the incidence of pyometra in bitches.

3.1.1. Breed

Information regarding breed of the animal diagnosed to be suffering from pyometra were analyzed to study the frequency distribution of pyometra in various breeds of bitches.

3.1.2. Age

The age of the bitch at the time of diagnosis of pyometra was obtained from each case record as well as from the clinical cases handled during the course of present investigation. On the basis of age the animals were grouped as less than 2 years, 2 to 5 years, 5 to 9 years, and more than 9 years. The frequency distributions of occurrences of
pyometra, in different age groups were compared to assess the possibility of predisposition of age of the animal and development of pyometra.

3.1.3. Parity of the animal

Data regarding the parity of the animals affected with pyometra was obtained to compare variation, if any, in the incidence of occurrence of pyometra between nulliparous, primiparous and pluriparous animals.

3.2. Prospective study

The Prospective studies were conducted on sixty female dogs which were presented during the course of present investigation. A tentative diagnosis of pyometra was made in these animals on the basis of Medical history, Clinical examination and Hematological studies.

3.2.1. Medical history

The medical history suggestive of pyometra included

1. History of clinical signs of illness following oestrus

2. Presence of vaginal discharges,

3. Abdominal distention

4. Clinical signs of illness such as depression, anorexia, polyurea, polydypsia, vomition and diarrhea

In addition to duration of illness, previous reproductive history if any, was also obtained.
3.2.2. Clinical examination

In every case confirmation of pyometra was based on a detailed clinical examination which comprised of

1. Recording of rectal temperature, pulse and respiratory rate.

2. Presence or absence of vaginal discharges, as evident by the visual observation of the discharges between vulval lips or soiling of the perineum and matting of perineal hairs, (Plate No. 1).

3. Changes in the color, consistency and volume of vaginal discharges,

4. Presence or absence of vulval edema.

5. Presence or absence of dehydration as judged by the loss of elasticity of the skin

Following clinical examination, each animal was subjected to gentle abdominal palpation for evidence of distended uterus suggestive of pyometra. Then they were subjected to transabdominal ultrasonography for confirmation of pyometra

3.2.3. Ultrasonographic studies

Dogs suspected for pyometra were subjected to ultrasonography using a 5 to 7.5 MHz trans-abdominal probe (HONDA ELECTRONICS, HS-2000). The ultrasonographic instrument used in the present study has been specifically designed for Veterinary Gynaecological cases. The diagnosis of pyometra is best made with the aid of ultrasonography and findings typically included an enlarged uterus with convoluted, tubular horns filled with anechoic to hypo echoic fluid. The luminal contents are usually homogenous, but the contents may also be echo dense with slow and swirling patterns. (Plate.No.2) .
Plate 1: Pussy vaginal discharge observed in a bitch with pyometra (Discharge came out during vaginoscopic examination).
Briefly, the procedure employed for ultrasonic measurements of two selected biometric parameters (external diameter of the uterine horn and thickness of the uterine wall) was as follows. The hair on the ventral abdomen between xiphi sternum and pubis and extending to several centimeters on either side of the midline was shaved and ultrasonography was carried with the patient in dorsal recumbency. After application of coupling gel to improve the contact, the sector probe was placed on the abdomen and slowly moved anteriorely and laterally in front of the bladder until the distended uterine horns were clearly visible. The image was frozen and the diameter of the uterine horn and thickness of the uterine wall were measured.

### 3.3. Treatment groups

The animals diagnosed for pyometra were randomly assigned to the following five treatment protocols with twelve animals in each group.

**Group I (Antiprogestin):** Bitches with pyometra assigned to this group were administered with antiprogestin at a dose rate of 10 mg/kg body weight orally once on the day of diagnosis (0day) and subsequently on day 1 and 2. The treatment was repeated on day eight.

**Group II (Antiprogestin + Prostaglandin F₂α):** Bitches under this group were administered with antiprogestine at the same dose and duration as in group I and additionally received injections of synthetic PGF₂α on day 1, 2, and 3 after the initiation of antiprogestin treatment twice daily at the dose rate of 10µg/kg body weight subcutaneously.
Plate 2: Ultra sound image of the caudal abdomen of a bitch with pyometra showing distended uterus with inflammatory exudates
**Group III (Antiprogestin + Prostaglandin E):** Each animal of this group received antiprogestine at a dose and duration similar to group I and additionally they also received PGE at the dose rate of 3µg/kg body weight on day 1, 2, & 3 once orally.

**Group IV (Prostaglandin F₂α):** Bitches assigned to this group received injections of synthetic PGF₂α on day 0, 1, 2, 3, and 4. twice daily at the dose rate of 10µg/kg body weight subcutaneously.

**Group V (Surgical):** Bitches assigned to this group were subjected to ovario hysterectomy. These animals were adequately stabilized by administration of intravenous fluids and broad spectrum antibiotics prior to surgery. The dogs were fasted overnight and they were pre medicated with diazepam (0.5 mg per kg body weight, IV) and Atropine sulphate (0.04 mg per kg body weight, IM) about 10 minutes prior to surgery. The ventral abdominal area extending from xiphoid to pubis prepared aseptically for midline incision. Further general anaesthesia was induced using Isoflurane (FORANE, Abbott Laboratories Ltd., England) as an inhalant anaesthetic agent. Isoflurane was administered through a face mask using a Boyle’s anesthetic apparatus initially at the rate of 3-5% until the animal attained the surgical plane of anesthesia. Simultaneously, oxygen was also infused at the rate of 1.5 %. Once the animal reached the surgical plane of anesthesia, it was maintained with Isoflurane at 1 % concentration and oxygen at 1.5 % concentration.

The distended uterus with ovaries was exteriorised. The two haemostats were applied on either side of ovary and ligature was applied around the ovary and artery using No1-0 chromic catgut. After ligation, the ovary was severed using a curved scissor. The
same procedure was repeated for the contra lateral ovary. The stumps were checked for bleeding points and returned to the abdomen. The broad ligaments were separated and the utero-ovarian vessels were ligated separately. The body of the uterus was clamped and ligated with No1-0 chromic catgut at the cervical end. The uterus was then transected. The stump was checked for bleeding and returned to the abdomen. The linea alba was opposed with simple interrupted sutures using No1-0 chromic catgut. The closure of the skin was done with horizontal mattress suture using No1-0 prolene non absorbable synthetic suture material.

All the animals of surgical and pharmacological treatment groups also received an antibiotic cover with ampicillin and cloxacillin at a dose rate of 10mg/kg body weight, orally from the day of initiation of treatment and antibiotics changed as soon as results of antibiotic sensitivity test (ABST) were obtained. Antibiotic treatment continued for three weeks. Animals also received fluids during the course of treatment to correct electrolyte and fluid imbalance if any.

3.4. Collection of clinical material

3.4.1. Collection of vaginal swabs

Animals were adequately restrained on an examination table and the perivaginal area was scrubbed with a potent antiseptic. Sterile cotton swab sticks (Avon Laboratories, Kolkata, India) were passed into the vagina with the vaginal labia parted with the help of vaginal speculum. The stick was directed cranio-dorsally initially (avoiding the clitoral fossa and the urethral orifices) and then longitudinally through a length of about 12 cm as described by Baba et al. (1983) and Olson et al.(1984). The swab was then rolled
around the vaginal wall gently and withdrawn carefully into its adequately labeled sterile container for transport to the laboratory.

3.4.2. Collections of blood samples

Peripheral blood samples were collected before, during and after the treatment (Day 0, 4 & 9) from the radial vein into Vacutainer tubes (AcCuvet, Quantum Biologicals Pvt Ltd, Chennai, India) with EDTA [ethylenediaminetetraacetic acid]. The blood samples were processed within 3–4 h after collection for the hematological parameters viz. hemoglobin (Hb), Packed Cell Volume (PCV), Total Leukocyte Count (TLC) Differential Leukocyte Count (DLC) performed on the day of blood collection.

Blood samples collected in sterile vials (AcCuvet, Plain Quantum Biologicals Pvt Ltd, Chennai, India) without anticoagulant (on day 0, 2, 4, 6, and 8) and the sera obtained were preserved at -20 ºC until subjected for estimation of progesterone. The blood serum samples obtained on day 0, 4 and 9 of the treatment were subjected for estimation of total protein, albumin, globulin, blood urea nitrogen, creatinine, SGPT, and alkaline phosphatase.

3.5. Laboratory procedure on vaginal samples

3.5.1. Culture and Sensitivity test (CST)

The vaginal samples were inoculated onto 5% sheep blood and McConkey agar and the plates incubated at 37ºC for 24-48 hours after which discrete colony growths were observed and recorded. Gram staining and other routine biochemical tests were
further employed to identify bacteria based on standard procedures (Carter, 1975 and Cheesbrough, 1994).

The antibiotic sensitivity pattern of vaginal swab samples of all the 60 female dogs of different treatment groups were carried out before initiation of treatment by standard disc diffusion technique described by Bauer et al. (1966).

The processed vaginal swab samples were inoculated into the nutrient broth and incubated at 37°C till the turbidity matched with Barium Sulphate standard and then the culture in the nutrient broth were seeded on fresh Muller Hinton agar plates. Antibiotic discs (HiMedia Laboratories Ltd., Bombay) were carefully placed on seeded M-H agar plates and pressed gently with forceps. The discs were placed at a minimum distance of 15 millimeter from the edge of plate and also from each other to avoid overlapping of the zone of inhibition. The plates were allowed to settle at room temperature for 30 minutes and incubated at 37 ºC for 16 to 18 hours.

The antibiotic sensitivity of the vaginal swab samples were assessed by measuring the zone of complete inhibition around the disc. Based on the diameter of Zone of inhibition, the sensitivity pattern of microorganisms to antibiotics were identified as sensitive or intermediate or resistant. Each vaginal swab samples from all the dogs in the different groups were subjected to culture and sensitivity test using standard discs of 20 antibiotics as detailed hereunder.
Plate 3: Antibiogram of vaginal samples collected from bitches with pyometra
### Zone size interpretive particulars of the antibiotics used (Plate No.3)

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Discs used</th>
<th>Strength (µg/disc)</th>
<th>Diameter of Zone of inhibition</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Sensitive (mm or less)</td>
</tr>
<tr>
<td>1</td>
<td>Amicacin</td>
<td>40</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>Amoxicillin</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>Ampicillin</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>Azithromycin</td>
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<tr>
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</tr>
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</tr>
<tr>
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<td>16</td>
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3.5.2. Haematological and Blood biochemical studies

The hematological and biochemical parameters were recorded using SYSMEX model pocH-100i and BIOSYSTEMS A15 respectively. The following hematological parameters studied were

a. Haemoglobin (Hb), (g/dl)
b. Packed Cell Volume (PCV),
c. Total Leukocyte Count (TLC) (cells/cmm)
d. Differential Leukocyte Count (DLC)

The blood biochemical parameters studied were

a. Blood Urea Nitrogen (gms %)
b. Serum Creatinine (gms %)
c. Alanine amino transferase (SGPT) (U/l)
d. Alkaline phospatase
e. Total protein
f. Albumin
g. Globulin

3.6. Serum progesterone estimation

The blood serum samples at -20 ºC were used for estimation of progesterone. The level of progesterone was measured by the competitive immunoassay method
(Immulite®, Los Angeles, CA, USA). The intra-assay coefficient of variation was 2.07 \% for progesterone. The lower limit of detection was 0.2 ng/ml for progesterone.

3.7. Evaluation of the bitches during and following different treatment protocols

All the animals in different groups were examined on the fourth day after the initiation of treatment and again on the ninth day for the clinical evidence of resolution of pyometra. The parameters studied were as follows:

1. Attitude and appetite of the animal
2. Colour, consistency and volume of vaginal discharges
3. Changes in the degree of distention of uterus as palpated per abdominally
4. Ultrasonographic evidence of resolution of pyometra as evident by the changes in the thickness of uterine wall, external diameter of the uterine horn and the uterine luminal contents (Plate No.4)
5. Changes in the hematological, biochemical and hormonal profiles
6. During the course of treatment all animals were closely observed for the signs of side effects such as vomition, diarrhoea, salivation, panting, urination, defecation and any other adverse reactions.

3.8. Evaluation of the therapeutic efficacy of different treatment protocols

Following completion of different treatment protocols, all the animals were observed for six months to assess the complete recovery or reoccurrence of the disease. The therapeutic efficacy for each treatment protocol was assessed by recovery rate, relapse rate and mortality rate.
Plate 4: Ultra sound image of the caudal abdomen of a bitch with pyometra showing gradual decrease in the volume of the distended uterus, during and after completion of treatment.
3.9. Statistical Analysis

The data generated from the clinical trials was tabulated and the mean and standard error were computed for all the groups. Wherever the values were expressed as percentage, they were subjected to arc sin transformation in order to stabilize variance utilized for analysis. One way analysis of variance was performed to test the variation between the groups and the group means were compared by least square significance difference test (LSDT) as per techniques of Snedecor and Cochran (1980). Comparisons were considered significantly different at $P<0.05$. 