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Concentrations of Inflammatory Markers and Clinical Findings in Bitches Surgically Treated for Pyometra in Khon Kaen, Thailand

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Concentrations of Inflammatory Markers and Clinical Findings in Bitches Surgically Treated for Pyometra in Khon Kaen, Thailand

Koncentrationer av inflammatoriska markörer och kliniska fynd hos tikar som behandlats kirurgiskt för pyometra i Khon Kaen, Thailand

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SUMMARY

Pyometra is a common reproductive disease affecting intact bitches. The disease is defined by accumulation of purulent material within the uterus, and can potentially be life-threatening. Clinically, affected bitches can present with a variety of signs and diagnosis can be challenging in more indistinct cases. It is not uncommon that bitches with pyometra manifest systemic inflammatory response syndrome (SIRS) in response to the bacterial infection, i.e. sepsis. Rapid initiation of treatment is then necessary in order to prevent progression into hypotension and organ dysfunction, known as septic shock. If one or more organ systems fail entirely, death usually follows.

Delay of effective antibiotic treatment after the onset of hypotension in patients diagnosed with septic shock has been shown to be a crucial therapeutic factor associated with mortality. Correct initiation of treatment is however complicated by the fact that it is diagnostically challenging to identify patients suffering from sepsis at an early stage. There is no gold standard to diagnose sepsis, since blood cultures may remain negative in 30-50 % of the patients and is not yet rapid enough. Clinical criteria used to detect SIRS in dogs include body temperature, heart rate, respiratory rate, white blood cell count and band neutrophils. These criteria are unspecific and when aiming for high sensitivity, the specificity decreased resulting in a relatively high proportion of dogs falsely diagnosed with SIRS. Consequently, there is a need for biomarkers to achieve effective diagnosis of sepsis in dogs, and make it possible to initiate necessary treatment at an early stage. This study investigates the possible use of albumin, iron and C-reactive protein (CRP) as adjunctive diagnostic markers for sepsis and prediction of prognosis measured as prolonged hospitalization.

Nine dogs with pyometra and 15 healthy control dogs were included in this study. Blood samples were collected pre-operatively, 24 hours post-operatively and 7-10 days post-operatively. The serum was analysed in Sweden for concentrations of albumin, iron and CRP. Tissue samples were removed from the uteri of the dogs surgically treated for pyometra and embedded in paraffin in Thailand. The embedded samples were then histologically examined in Sweden. Blood cultures and bacterial swabs from the uteri were analysed for bacterial growth and isolated strains tested for antimicrobial sensitivity in Thailand.

The results of this study showed that none of the investigated inflammatory markers differed between septic and non-septic bitches, which indicate that none of the investigated parameters have a potential clinical value for detection of sepsis. However, the number of dogs included in the study was low and a larger study material would be useful for further evaluation. Broad-spectrum antibiotics was used routinely in bitches treated surgically for pyometra and also in bitches admitted for elective spaying at Khon Kaen University Animal Hospital. Multiresistance could not be observed in any of the isolates from the uteri bacterial swabs, but a larger study material and a standard profile of antimicrobials tested would be beneficial to give a more correct interpretation of the antimicrobial susceptibility tests and to survey antibiotic resistance.

SAMMANFATTNING

Pyometra är en vanligt förekommande reproduktiv sjukdom som drabbar intakta tikar. Sjukdomen definieras av ansamling av purulent material i livmodern, och kan potentiellt vara livshotande. Drabbade tikar kan kliniskt uppvisa en variation av sjukdomstecken, och diagnos av sjukdomen kan vara utmanande i mer indistinkta fall. Det är inte ovanligt att tikar med pyometra manifesterar systemic inflammatory response syndrome (SIRS) till följd av den bakteriella infektionen, det vill säga sepsis. Snabb initiering av behandling är då nödvändigt för att förhindra utvecklande av hypotension och organsvikt, även känt som septisk chock. Om ett eller flera organsystem fallerar helt leder detta ofta till döden.

Fördröjning av antibiotikabehandling efter hypotension inträtt hos patienter med septisk chock har visat sig vara en betydande terapeutisk faktor associerad med dödlighet. Korrekt behandling i tid kompliceras dock av att det är diagnostiskt utmanande att identifiera patienter med sepsis på ett tidigt stadie. Det finns ingen optimal standardmetod för att diagnostisera sepsis, då blododlingar kan vara negativa i 30-50 % av fallen och ännu inte är tillräckligt tidseffektiva. Kliniska kriterier som används för att diagnostisera SIRS hos hundar inkluderar kroppstemperatur, hjärtfrekvens, andningsfrekvens, antal vita blodceller och bandneutrofiler. Dessa kriterier är ospecifika och utvecklade med hög sensitivitet, vilket resulterar i en relativt hög andel hundar som blir falskt diagnostiserade med SIRS. Detta visar att det finns ett behov av biomarkörer för att effektivt kunna diagnostisera sepsis hos hundar och möjliggöra insättning av behandling i ett tidigt stadium. Syftet med den här studien var att utvärdera möjlig användning av albumin, järn och C-reaktivt protein (CRP) som diagnostiska markörer och för att förutsäga prognos mätt som förlängd sjukhusvistelse.

Nio hundar med pyometra och 15 friska kontrollhundar inkluderades i studien. Blodprover samlades innan operation, 24 timmar efter operation och 7-10 dagar efter operation. Serum analyserades i Sverige avseende koncentrationer av albumin, järn och CRP. Vävnadsprover togs från livmödrarna som avlägsnats vid operation av hundarna med pyometra och preparerades i Thailand. De preparerade vävnadsproverna undersöktes sedan histologiskt i Sverige. Blododlingar och bakterieodlingar från livmödrarna analyserades och testades för antimikrobiell känslighet i Thailand.

Resultaten av den här studien visar att ingen av de inflammatoriska parametrarna som undersöktes skiljde sig mellan septiska och icke-septiska hundar, vilket indikerar att ingen av dessa parametrar har potentiellt kliniskt värde för diagnostik av sepsis. Dock var antalet hundar i denna studie lågt, och ett större studiematerial skulle vara fördelaktigt för vidare utvärdering. Bredspektrumantibiotika användes rutinmässigt till tikar som opererades för pyometra eller kastrerades på Khon Kaen University Animal Hospital. Multiresistens kunde inte påvisas i något av isolaten från bakteriesvabbarna, men ett större studiematerial och en standardprofil för vilka antibiotikapreparat som testades skulle vara fördelaktigt för en mer korrekt tolkning av känsligheten mot antibiotika och för att kartlägga antibiotikaresistens.

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Abbreviations

ALP	Alkaline phosphatase
CRP	C-reactive protein
ICU	Intensive care unit
KKU	Khon Kaen University
LODS	Logistic organ dysfunction syndrome
MODS	Multiple organ dysfunction syndrome
MFS	Minor field study
OHE	Ovariohysterectomy
PCV	Packed cell volume
RBC	Red blood cell count
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential organ failure assessment
SLU	Swedish University of Agricultural Sciences
UDS	University Animal Hospital
WBC	Total white blood cell count

INTRODUCTION

Pyometra is a potentially life-threatening disease, which commonly affects middle age and older bitches (De Bosschere, 2002). The disease is defined by accumulation of purulent material within the uterus, causing a variety of local and systemic symptoms, and requiring surgical or medical intervention in order to resolve (Dow, 1959; Hagman *et al.,* 2011b). In countries where spaying of healthy dogs is not generally practised, 23-24% of the bitches will have experienced pyometra by 10 years of age (Egenvall *et al.,* 2001). Similar results were presented in a more recent study, where the proportion of the bitches that had developed pyometra at 10 years of age was 19 % (Jitpean *et al.,* 2012). The pathogenesis of pyometra is still not completely understood, despite decades of research on the subject, but it is generally considered that bacteria ascending from the normal vaginal flora proliferate in a progesterone-primed uterus and establish an infection (Hagman, 2012). The most frequently isolated bacteria in cases of pyometra are *Escherichia coli* (*E.coli*) (Bigliardi *et al.,* 2004; Dow, 1959; Wheaton *et al.,* 1989).

Clinically, the bitch with pyometra may present with a variety of signs including vaginal discharge, polydipsia, polyuria, anorexia, depression, vomiting and lethargy (Bigliardi *et al.,* 2004; Jitpean *et al.,* 2014a; Kaymatz *et al.,* 1997; Martins *et al.,* 2015). In characteristic cases identification and diagnosis of the disease is undemanding, but the diagnosis may be challenging in cases where vaginal discharge is absent (closed-cervix), and when the clinical presentation or history are indistinct (Hagman, 2012). Useful diagnostic tools for the disease include abdominal radiographs (Wheaton *et al.,* 1989) and ultrasound investigation (Bigliardi *et al.,* 2004). Ovariohysterectomy (OHE) is generally recommended as the treatment of choice for canine pyometra, especially when the bitch lacks significant reproductive value or the owner has no strong wish to breed the bitch (Rootwelt-Andersen & Farstad, 2006).

It is not uncommon for bitches with pyometra to manifest systemic inflammatory response syndrome (SIRS) associated with the bacterial infection, i.e. sepsis (Fransson *et al.,* 2007). Septic shock follows when the syndrome proceeds into hypotension and organ dysfunction (Parrillo, 1993). If one or more organ system fails entirely, death usually follows (Parrillo, 1993). Currently, there is no rapid and exact test to diagnose sepsis, which is why certain clinical criteria are used to pinpoint patients more prone to suffer from sepsis (Hauptman *et al.,* 1997; Seymour *et al.,* 2016). Since there is no gold standard for diagnosing sepsis (Seymour *et al.,* 2016), there is a necessity to identify biomarkers to achieve effective diagnosis of dogs suffering from sepsis, allowing rapid initiation of appropriate therapy (DeClue *et al.,* 2011). Many potential biomarkers have been evaluated for use in sepsis, but none of them has so far been proven to have sufficient specificity or sensitivity to be regularly used in clinical practise (Pierrakos & Vincent, 2010).

The aim of this study was to investigate the levels of albumin and iron in bitches diagnosed with pyometra in order to evaluate the values of these measurements in predicting outcome and identifying bitches developing sepsis as a complication to the disease, and to compare with the more routinely used marker for systemic inflammation, i.e. the acute phase protein C-reactive protein (CRP). Additionally, the study provided an opportunity to compare standard antibiotic treatment in Sweden versus Thailand, as well as bacterial species and existent antibiotic

resistance patterns in the isolates commonly causing uterine infection in dogs treated surgically for pyometra at Khon Kaen University Animal Hospital, Thailand.

LITERATURE REVIEW

Pyometra

History and prevalence/incidence

In a retrospective cross-sectional study in the UK performed by Gibson *et al.,* (2013) with a total of 1728 cases of pyometra, the overall period prevalence of pyometra in the female dog population was 2.2 %, which is similar to previously reported values (Egenvall *et al.,* 2001; Whitehead, 2008). Factors associated with an increased risk of developing pyometra include nulliparity (Hagman *et al.,* 2011; Niskanen & Thrusfield, 1998) and exogenous estrogen therapy in young bitches (Niskanen & Thrusfield, 1998; Whitehead, 2008). In a breed-matched case control study, Hagman *et al.* (2011b) found that previous pregnancy was a factor that had protective effect against pyometra in some breeds, which indicates a breed-variation concerning protective- and risk-factors.

Certain breeds have been reported to have a predisposition for pyometra, specifically Rough Collie, Rottweiler, Cavalier King Charles Spaniel and Golden Retriever (Egenvall *et al.,* 2001; Niskanen & Thrusfield, 1998). The Bernese Mountain Dog was shown to have the highest rank for development the disease in a study including 110 breeds (Jitpean *et al.,* 2012). Breeds with a low risk of developing pyometra include Drevers, German Shepherd Dogs, Swedish Hounds and Dachshounds (Egenvall *et al.,* 2001). However, in the study by Niskanen & Thrusfield (1998) no pedigree dog had a significantly reduced crude risk, but there was evidence for a reduced risk in mongrels. The notable variations between breeds regarding the incidence of pyometra indicate that genetic factors may predispose and/or protect for development of the disease (Jitpean *et al.,* 2012).

Ethiology and pathogenesis

The pathogenesis of pyometra is still not yet entirely understood. Hormonal influence on the uterus was suspected to be important in the pathogenesis in pyometra in early reports, since the disease was most commonly observed during the influence of progesterone in diestrus (Dow, 1959). Progesterone has been shown to 1) stimulate endometrial gland secretion; thus providing a suitable environment for bacterial growth, 2) suppress immune responses, 3) cause functional closure of the cervix, which prevents drainage of contents of the uterus, and 4) mediate cystic endometrial hyperplasia (CEH) (Chaffaux & Thibier, 1987; Austad *et al.,* 1979). Early reports suggested that prolonged exposure to progesterone caused susceptibility to pyometra, which was shown by experimentally inducing the disease by admission of exogenous progesterone to ovariectomized bitches, (Dow, 1959). When measuring concentrations of progesterone in blood plasma in bitches suffering from pyometra, Caffaux & Thibier (1978) could not detect any prolonged or extensive secretion of progesterone in these bitches, contradicting to what the experiments by Dow (1959) would suggest. In a more recent Finnish study, no significant risk-enhancing effect of progestin-treatment (medroxyprogesterone acetate) was observed and it was concluded that such risk, if present, is probably low (Niskanen & Thrusfield, 1998). These contradictions could be due to the fact that the bitches in the experimental study by Dow (1959)

received large and repeated doses of progesterone, resulting in plasma concentrations far exceeding the physiological and therapeutic levels. Additionally, the number of dogs treated with progestin in the study by Niskanen & Thrusfield (1998) was low.

Other investigations have evaluated the exaggerated response to progesterone due to upregulation of endometrial hormone receptors, potentially causing development of the disease (De Bosschere *et al.,* 2002). It has been shown that expression of estrogen and progesterone receptors are elevated in uteri of bitches with CEH, but not in bitches with pyometra (De Bosschere *et al.,* 2002).

In the common dogma, degenerative changes in the uterine tissue associated with CEH (cystic expansion of glands, fibrosis etc.) are proposed to provide suitable conditions for establishment of uterine infection. Opportunistic pathogens invade the already compromised uterus by an ascending route of infection from the vagina or faecal flora, proliferate and establish infection within the uterus. The infection is considered to be established due to presence of crypts and cysts where bacteria are able to proliferate, an excessive amount of secretory fluids accumulated in the lumen of the uterus, and reduced local immunity, either related to or resulting from local tissue degeneration of the uterus. This concept is known as the "Cystic Endometrium-Pyometra Complex". (De Bosschere *et al.,* 2001; De Bosschere *et al.,* 2002). However, in contrast to earlier reports, it was noted in the study by De Bosschere *et al.,* (2001) that CEH and pyometra often occur independently, indicating that it is probably more correct to divide the CEH-pyometra complex into two entities.

The most frequently isolated bacteria in cases of pyometra are *Escherichia coli (E. coli)* (Fransson *et al.,* 1997; Bigliardi *et al.,* 2004; Dow, 1959; Sandholm *et al.,* 1975). Other bacteria isolated from the uterus in bitches with pyometra include *Streptococcus* spp., *Pseudomonas* spp., *Proteus* spp., and *Klebsiella* spp. (Fransson *et al.,* 1997; Bigliardi *et al.,* 2004). *Escherichia coli* has been shown to adhere specifically to receptors in progesterone-stimulated endometrium, thus giving one explanation for the observed predominance of this infecting agent (Sandholm *et al.,* 1975). In a study by Arora *et al.* (2006) aiming to develop a trustworthy model for the study of the cystic endometrial hyperplasia and pyometra complex (CEH/P) in the bitch, a culture of *E. coli* (with five uro-pathogenic virulence factors: pap, sfa, hlyA, cnf1 and fim) was inoculated intra-vaginally daily in one group of ovariectomized bitches throughout stimulated estrus, and directly into the uterus on stimulated diestrus in a second group of ovariectomized bitches. The results of the study indicate that intra-uterine inoculation of *E. coli* under given conditions can reliably induce CEH/P, whilst the intra-vaginal inoculation failed to induce CEH/P. The result of the intra-vaginal inoculation is similar to earlier findings where intra-vaginal inoculation of *E. coli* during naturally occurring estrus also failed to induce CEH/P, which suggests that other factors than the presence of infecting agents might be required to cause infection and contribute to the persistence and action of bacteria in the uterus (Nomura *et al.,* 1988).

Diagnosis

Findings during physical examination

In a bitch with pyometra, a variety of clinical signs including vaginal discharge, polydipsia, polyuria, anorexia, depression, vomiting and lethargy may be present (Bigliardi *et al.,* 2004; Jitpean *et al.*, 2014a; Kaymatz *et al.,* 1997; Martins *et al.,* 2015). Other physical examination abnormalities that have been noted are hyperthermia and abdominal pain on palpation (Fransson *et al.,* 2007). Some degree of vaginal discharge is evident in most cases, in a variety of forms; from seromucous to purulent, bloody or with a chocolate-like appearance usually with bad odour (Kaymatz *et al.,* 1997). Several of the common clinical signs reported, such as polyuria/polydipsia, anorexia and vomiting, reflects the systemic character of the disease (Jitpean *et al.,* 2014).

Clinically, pyometra can be classified as open-cervix or closed-cervix. Vaginal discharge indicates that the cervix is open, and thus the uterine content is being drained (Martins *et al.,* 2015). The diagnosis of the disease may be challenging in less characteristic cases where vaginal discharge is absent (closed-cervix), and when the clinical presentation or history are indistinct (Hagman, 2012).

Cytological examination

Vaginoscopic examination and preparation of cytologic samples can help determine vaginal discharge in cases where the bitch presents with such. In vaginal smears from bitches with open-cervix pyometra, the presence of cell degenerations have been noted, such as deteriorated cytoplasmic wall, alterations and karyolysis in epithelial cells. An increase in white blood cell (WBC) numbers has also been observed, with a large amount of degenerated and normal leukocytes and frequently degenerated neutrophils. However, in bitches with closed cervix pyometra, absence of leukocytes on the cytological samples has been noted. Basal and parabasal cells have shown to be the dominant vaginal smear cell type in vaginal cytology of pyometra cases. (Kaymaz *et al.*, 1999)

Blood chemistry and hematological findings

Common findings in bitches with pyometra include anemia, leukocytosis, and presence of band neutrophils (Fransson *et al.,* 1997; Jitpean *et al.,* 2014a; Maretta *et al.* 1989). Fransson *et al.* (1997) discovered that bitches infected with Gram-negative bacteria (GNB) hade higher total WBC than other groups of bitches in the study. The differential cell count also showed noticeable differences, since bitches with GNB in the uterus had a more pronounced reaction, exhibited as a left shift, in WBC compared to the other groups. Additionally, toxic granulation of neutrophils was only seen in bitches where GNB had been isolated from the uterus. Some cases of pyometra will, however, display leukopenia, which has been shown to be associated with a poorer prognosis and may be used as a mean to predict outcome and prolonged post-operative hospitalization (Jitpean *et al.,* 2014a). The mild to moderate normocytic, normochromic anemia is considered to reflect the chronic nature of the disease with toxic suppression of the bone marrow, lack of accessible iron and loss of erythrocytes to the uterus. The evaluation of anemia can be affected due to dehydration (Hagman, 2012).

Other reported laboratory findings include increased levels of alkaline phosphatase (ALP) (Fransson *et al.,* 1997), serum creatinine (Martins *et al.,* 2015) and increased levels of gamma globulins, reflecting as hyperproteinemia (Sandholm *et al.,* 1975). Changes in electrolyte levels

and acid-base balance are also common findings in bitches with pyometra (Hagman, 2012; Kaymaz *et al.*, 1999).

Diagnostic imaging

Abdominal radiographs have been shown to demonstrate an enlarged uterus in 80-90 % of the cases, and are consequently being considered a useful diagnostic tool for pyometra (Wheaton *et al.*, 1989). Ultrasonographic investigation is also a useful and trustworthy method to detect pathological alterations in the uterus. Ultrasonographically, it is possible to clearly evaluate endometrial integrity, uterine wall thickness, uterine expansion, cystic endometrial glands and presence of exudates such as blood, mucus and pus (Bigliardi *et al.*, 2004).

Treatment

Surgical Treatment

Ovariohysterectomy (OHE) is generally recommended as the treatment of choice for canine pyometra (Rootwelt-Andersen & Farstad, 2006). The surgical procedure removes the source of infection, and prevents recurrence (Hagman, 2012). In a survey among Norwegian small animal practitioners it was shown that the general health in the bitch influenced the choice of treatment in nearly 90 % of the cases. The breeding status, presence of an open versus closed cervix, expected prognosis and the owners' capacity and willingness to pay for the procedure were factors influencing the choice of treatment in about 50 % of the cases, whereas the age of the bitch only affected the decision in 15 % of the cases (Rootwelt-Andersen & Farstad, 2006). Surgical complications that have been reported include anesthetic difficulties, hemorrhage, peritonitis, uterine torsion, inadequate removal of the ovaries, ureteral trauma, uterine stump pyometra, incisional swelling, wound infection and fistulous tracts (Maretta *et al.* 1989).

The risk of complications due to surgery and anesthesia can be reduced if patients in poor condition receive appropriate therapy to stabilize their condition pre-surgery (Fransson & Rangle, 2003). According to the current standard routines used at the Swedish University of Agricultural Science (SLU, 2016), the use of intravenous administered broad-spectrum antibiotics is recommended at the time of induction to reduce possible bacteremia caused by manipulation of the uterus; the dose ought to be repeated in cases where the operation lasts more than 90 minutes. Post-surgical antibiotic therapy should however be used solely in systemically ill bitches or bitches with complications present or increased risk of developing complications.

The mean survival rate among Norwegian bitches treated surgically for pyometra has been reported to be 96 % (Rootwelt-Andersen & Farstad, 2006). In a more recent Swedish study, the postoperative death in bitches surgically treated for pyometra by OHE was 1 % (Jitpean *et al.*, 2014a).

Medical treatment

In Sweden, medical treatment of pyometra is seldom performed and is conducted only when the general condition of the bitch is slightly or moderately affected (Ros *et al.*, 2014). This differs from other countries where medical treatment is also performed when the bitch is in poor general condition (Fieni *et al.*, 2014). However, it is contraindicated with medically

treatment in bitches that display signs of systemic illness or suspected peritonitis (Fieni *et al.*, 2014). Drugs that can be used for medical treatment of pyometra include progesterone-receptor antagonists, prostaglandins and dopamine agonists, or different combinations of these drugs (Fieni *et al.*, 2014) in combination with antimicrobials (Ros *et al.*, 2014). Regardless of the chosen specific medical treatment, a general treatment including fluid therapy, antibiotics to prevent septicaemia, and the use of an Elizabethan collar to inhibit the bitch from ingesting vulvar discharge should be applied in all cases (Fieni *et al.*, 2014).

Agleprisone is an antiprogestin, and a progesterone receptor antagonist. Repeated administration of agleprisone with or without additional treatment with low doses of prostaglandins is considered to be the most effective medical management of pyometra, with minor side effects (Fieni *et al.*, 2014). In a Swedish study, the overall success rate (defined as return to clinically healthy status) of treating pyometra medically with aglepristone was 75 % (Ros *et al.*, 2014). The recurrence rate in the same study was reported to be 48 %, with a follow-up period of 6 years. In a survey among Norwegian veterinarians, the mean survival rate in bitches treated medically for pyometra has been reported to be 74 % on a short-term basis (throughout the affected cycle) (Rootwelt-Andersen & Farstad, 2006). With regard to future breeding, the prognosis on long-term basis was 37 % (Rootwelt-Andersen & Farstad, 2006). Mating the bitch at the first estrus post-treatment has been recommended (Fieni *et al.*, 2014), since pregnancy has been shown to have a somewhat preventive effect on uterine disease (Niskanen & Thrusfield, 1998) and the risk for recurrence is evident.

Recommendations regarding antibiotic treatment of several conditions in dogs, including pyometra, was formed by an expert group consisting of clinical pharmacologists, microbiologists and clinicians with specialist competency regarding diseases in dogs at an expert meeting arranged by the Swedish Medical Products Agency on the 10-11th of March 2016. According to these recommendations, evidence supporting positive effects of antibiotics treatment in addition to the hormonal treatment is lacking. Therefore, no recommendations are given regarding preparations, doses and length of treatment (Läkemedelsverket, 2016). This differs from previous recommendations in the antibiotic policy revised by the Swedish Veterinary Association where, whilst waiting for results from the bacteriological culture, drugs that are effective against Gram-negative bacteria were recommended, in combination with antiprogestins and/or prostagandin treatment. Weekly follow-up appointments were recommended to determine appropriate length of the antibiotic treatment (Sveriges Veterinärförbund, 2009).

Prognosis

In a retrospective cross-sectional study by Gibson *et al.* (2013) the mean mortality rate of dogs surgically treated for pyometra at Greater Manchester Animal Hospital (GMAH) was 3.2 % (range 2.0-5.2 %). This result is similar to the 5 % mortality reported by Wheaton *et al.* (1989). In a study by Jitpean *et al.* (2014a) four bitches died postoperatively (in total, 315 bitches were surgically treated by OHE) resulting in a mortality of 1 %, which is slightly lower than in previous reports. However, the total mortality (including euthanasia) in the study by Jitpean *et al.* (2014b) was 10 %, which is higher compared to corresponding case fatality (death and euthanasia) of 4.3 % reported by Egenvall *et al.* (2001). This can to some extent be explained

by the fact that Egenvall *et al.* (2001) only included bitches <10 years of age, while the study of Jitpean *et al.* (2014a) included data from bitches of all ages. This difference in inclusion of data matters, since the owner might be more likely to decide upon elective euthanasia in an older bitch, and the likelihood of other diseases is higher in an older bitch.

Although the mortality rate of dogs suffering from pyometra is relatively low, morbidity throughout the period of the disease, surgery and hospitalization also have welfare consequences (Gibson *et al.*, 2013). A 3.5-fold increased risk for prolonged hospitalization and an 18-fold increased risk of peritonitis associated with leukopenia compared to normal WBC was discovered by Jitpean *et al.* (2014a), thus making leukopenia the most significant clinical biomarker identified in the study. Compared to leukocytosis, a WBC within normal range was associated with increased risk for prolonged hospitalization and/or peritonitis. Other factors that correlated with increased risk for prolonged hospitalization included moderately to severe depressed general condition in the bitch, pale mucous membranes, and elevated lactate levels (Jitpean *et al.*, 2014a).

Sepsis

Definition of sepsis, SIRS and MODS

There are several diseases that provoke a stimulus initiating production and release of circulating mediators triggering systemic inflammatory changes. The body's reaction to this cascade of inflammatory mediators is known as the systemic inflammatory response syndrome (SIRS) (Purvis & Kirby, 1994). The cycle commences with a local inflammatory response that occurs as a result of exposure to initiating stimuli, such as endotoxins from invading bacteria. This in turns leads to target cell activation and production of inflammatory mediators. The released mediators continue to function as target cell activators, even in the absence of the initiating agent, thus causing a escalating cycle of systemic inflammation (Purvis & Kirby, 1994). When SIRS is caused by infection, such as in pyometra, it is defined as sepsis (Purvis & Kirby, 1994). Regardless of the underlying disease, the advancement and complications are the same once the mediators have been released in the circulation, i.e., increased capillary permeability, peripheral vascular dilation and decreased cardiac function (Purvis & Kirby, 1994). Septic shock follows when the syndrome results in hypotension and organ dysfunction (Parrillo, 1993) The progression to organ dysfunction is known as multiple organ dysfunction syndrome (MODS) (Purvis & Kirby, 1994).

Septic shock

The presence of microorganisms and their toxins, initiating an inflammatory cascade, is what differs the pathogenesis of septic shock from other forms of shock. The process commences with proliferation of the microorganisms (Gram-positive or endotoxin-containing Gram-negative) at a nidus of infection. When proliferating locally, the organisms release various substances into the blood stream, including endotoxins (released from disrupted cell walls of Gram-negative bacteria during rapid growth or death). The microorganisms can also invade the blood stream directly, which can be detected in positive blood cultures. (Parrillo, 1993)

However, blood cultures may remain negative in many patients with sepsis (30-50 % of the cases) because of, for example, inadequate sampling or antibiotic pre-treatment (Linder *et al.*, 2010). The substances originating from the microorganisms stimulate release of endogenous mediators of sepsis from plasma precursors or cells (endothelial cells, neutrophils, monocytes or macrophages, among others). In humans, the organ systems commonly affected by these mediators include the lungs, kidneys, liver, heart, central nervous system and coagulation system. If one or more organ systems fail entirely, death usually follows. (Parrillo, 1993)

Sepsis criteria in human medicine

Currently, there is no rapid and exact test to diagnose sepsis, which is why certain clinical criteria are used to pinpoint patients more prone to suffer from sepsis (Hauptman *et al.*, 1997). Several clinical criteria to identify patients at risk of developing sepsis have been evaluated by Seymour *et al.* (2016). These criteria included systemic inflammatory response syndrome (SIRS), logistic organ dysfunction system (LODS), sequential [sepsis-related] organ failure assessment (SOFA) score, and the quick sequential [sepsis-related] organ failure assessment (qSOFA) score. qSOFA is the most recent developed scoring system, including Glasgow Coma Scale (GCS) score (Gill *et al.*, 2005) of 13 or less, respiratory rate of 22 breaths per minute or more, and systolic blood pressure of 100 mm Hg or less.

Among patients in the intensive care unit (ICU) with suspected infections, SOFA and the more complex LODS had statistically greater predictive validity when compared to SIRS criteria. The predictive validity for in-hospital mortality of SOFA did not differ significantly from LODS, but was strategically superior to SIRS and qSOFA. However, outside of the ICU, the more simple model qSOFA had statically greater predictive value compared to the SOFA score. Throughout evaluation under varied measurement conditions, different settings and localizations, the predictive validity of qSOFA remained robust. Corresponding to these results, The Third International Consensus Definitions Task Force recommended use of the SOFA score as criteria for sepsis when encountering ICU patients with infection. In non-ICU, the use of q-SOFA was recommended to prompt consider the likelihood of sepsis. (Seymour *et al.*, 2016)

Diagnosis of sepsis in dogs

To the author's knowledge, neither SOFA, qSOFA nor LODS have yet been adjusted or evaluated for use in dogs. However, the clinical criteria formerly used in humans to detect SIRS have been altered for use in dogs (Hauptman *et al.*, 1997). These criteria include fever or hypothermia, tachycardia, tachypnea, increased or decreased WBC, and/or increased per cent band neutrophils.

The limits of the clinical criteria for SIRS in dogs was first presented by Purvis & Kirby (1994), stating that the response is manifested by two or more of the following criteria: body temperature (BT) >103°F (39.4°C) or <100°F (37.8°C), heart rate (HR) > 160 beats/min, respiratory rate (RR) > 20 breaths/min, WBC count >12.000/μL, <4000/ μL, or >10% band neutrophils. The limits were slightly altered by Hardie (1995), which suggested BT >104.0°F (40.0°C) or <100.4°F (38.0°C), HR >120 beats/min, RR >120 breaths/min, WBC count >18.000/μL, <5000/ μL, or >5% band neutrophils. This led to investigations of sensitivity and

specificity of the proposed limits for recognition of SIRS by Hauptman *et al.,* (1997), among others. In the study by Hauptman *et al.,* (1997) it was shown that earlier suggested limits had a sensitivity of 77-83 % for the detection of SIRS. By further modifications of the limits for the criteria, the research group managed to reach a sensitivity of 97 %: BT >102.6°F (39.2°C) or <100,6°F (38.1°C), HR >120 beats/min, RR >20 breaths/min, WBC count >16.000/µL, <6000/ µL, or >3% band neutrophils, thus decreasing the risk of failure to detect SIRS before onset of MODS. However, when aiming for high sensitivity, the specificity decreased, resulting in a relatively high proportion of dogs falsely diagnosed with SIRS. Even if these criteria may help the clinician identify SIRS, they do not differentiate infectious from non-infectious causes of the illness (Declue *et al.,* 2011).

Prognosis

In a study by Fransson *et al.* (2007) including 53 cases of canine pyometra and 19 healthy control bitches, SIRS was present in 57 % of the bitches with pyometra. The mortality rate among SIRS positive bitches was low (3.3 %), which indicates that progression to MODS seldom occurs in surgically treated cases of pyometra. A positive SIRS status was related to increased morbidity and associated with prolonged hospitalization. In another study including 114 dogs with sepsis secondary to gastrointestinal leakage, the mortality rate was 70 % for dogs with MODS and 25 % for the dogs not yet developed MODS (Kenney *et al.,* 2010). The mortality rate increased with increasing number of affected organ systems (Kenney *et al.,* 2010).

Delay of effective antibiotic therapy after the onset of hypotension in human patients diagnosed with septic shock is a critical therapeutic factor associated with mortality according to data presented in a study by Kumar *et al.* (2006). The study was retrospective and included medical records of 2.731 adult patients with septic shock. If efficient antimicrobial treatment was delayed by even one hour following the commencement of hypotension related with septic shock, the mortality rate increased significantly, and for every additional hour to initiation of antimicrobial therapy in the first 6 hours following onset of hypotension, the survival rate dropped an average of 7.6 %. These findings shows the necessity of rapid initiation of adequate treatment in septic patients, and consequently the requirement of diagnostic tools to clinically identify patients suffering from sepsis at an early stage.

Biomarkers

The immediate identification of sepsis is quite difficult, since the most commonly used methods for finding the microorganisms causing sepsis (including cytology, culture, serology or histopathology) are not yet rapid enough (DeClue *et al.,* 2011). There is no gold standard for diagnosing sepsis (Seymour *et al.,* 2016; Linder *et al.,* 2010) since blood cultures may be negative in 30-50 % of the cases (Linder *et al.,* 2010). Consequently, there is a need for biomarkers to achieve effective diagnosis of dogs suffering from sepsis, allowing rapid initiation of appropriate therapy. Prognostic biomarkers are essential both for management of the individual dog as well as the population as an entity (DeClue *et al.,* 2011).

A literature review, including 3370 studies that evaluated 178 biomarkers, indicated that there are a number of biomarkers that can be used in sepsis, but none of those examined has sufficient

specificity or sensitivity to be regularly used in clinical practice (Pierrakos & Vincent 2010). A number of biomarkers have been evaluated for sepsis and/or pyometra in dogs, including C-reactive protein (Fransson *et al.*, 2007; Jitpean *et al.*, 2014), Serum amyloid A (Jitpean *et al.*, 2014), serum insulin growth factor-I (Jitpean *et al.*, 2014), tumor necrosis factor α (Fransson *et al.*, 2003), interleukin-6 (Fransson *et al.*, 2003) and NT-pCNP (DeClue *et al.*, 2011), among others.

In addition to help identify or rule out sepsis, a valuable biomarker should also manage to be used when guiding therapy. When considering the complexity of the response to sepsis, it seams unlikely that a singular ideal biomarker will ever be sufficient. Consequently, a combination of several biomarkers might be more efficient, but this demands further investigations. (Pierrakos & Vincent 2010)

Iron

Iron plays an important role in host defence and pathogen virulence. It is fundamental for both the host and the pathogen, since both require iron as a cofactor for essential enzymes involved in several elementary cellular functions and metabolic pathways. Free iron is toxic to cells, causing protein denaturation, lipid peroxidation and DNA damage as well as catalyses the formation of free oxygen radicals. Consequently, most iron in the host is only available when bound to specific proteins and free iron is relatively unobtainable. (Shaible & Kaufmann, 2004)

Most commonly, serum iron concentration is prominently decreased during systemic infection or inflammation, as contribution in non-specific host defence mechanism against pathogens (Cartwright *et al.*, 1946). The inflammatory response induced by pyometra in dogs has been shown to cause decreased systemic concentrations in iron. However, studies evaluating iron concentrations in response to infection in dogs with pyometra are rare. (Jitpean *et al.*, 2014c)

Albumin

Albumin is a multifunctional protein. The protein is negatively charged and has high intravascular concentration, which is the basis for its function as a main regulator of intravascular volume, homeostatic properties and transport tasks. Additionally, it has antioxidant properties, endotoxin inactivation capacity, immunomodulatory function and a potential role as an endothelial stabilizer. (Quinlan *et al.*, 2005).

Hypoalbuminaemia has been reported as an effect of Gram-negative infection and bacteremia in dogs and cats (Greiner *et al.*, 2008). Thus, it is not surprising that pyometra have been shown to induce hyperproteinemia in dogs (Jitpean *et al.*, 2014a; Kaymaz *et al.*, 1999). Concentrations of albumin did not differ significantly between bitches with or without sepsis in the study by Jitpean *et al.* (2014a), a finding that was unforeseen since lower concentrations of albumin have been reported in dogs with sepsis (Greiner *et al.*, 2008). However, only few studies has explicitly investigated albumin as a marker for sepsis in dogs (Jitpean *et al.*, 2014a).

C-reactive protein

C-reactive protein (CRP) is a cyclic pentamer that recognize foreign molecules and altered self, thus binding to a number of different pathogenic microorganisms or intracellular antigens of damaged cells (Murata *et al.,* 2004). It was first discovered in serum in patients with pneumococci infections (Tillet and Francis, 1930) and was later named for its ability to bind to polysaccharide C fraction from pneumococci (Eckersall & Connor, 1988). CRP was the first identified protein that increased rapidly at the onset of infection or inflammation, thus recognized as the first acute phase protein (Eckersall & Connor, 1988). Canine CRP was identified by its cross-reactivity in a latex agglutination test for human CRP, and shown to act like an acute phase protein analogous to the human CRP (Dillman & Coles, 1966; see Caspi *et al.,* 1984). Isolation and characterization of the canine CRP were performed by Caspi *et al.* (1984) showing for the first time that the molecule is a glycoprotein, with two of the five subunits glycosylated, unlike human CRP. A prominent feature of the canine CRP is a rapid manifestation and increased concentration in serum subsequent to an acute stimulus (Caspi *et al.,* 1984).

CRP is now extensively used as an indicator of infection and recovery from such (Du Clos & Mold, 2001). However, CRP is mainly produced in the liver as a response to cytokines released by macrophages including mainly IL-6 and IL-1 (Mold *et al.,* 2002) and is therefore better used as an indicator reflecting the extent of acute inflammation in different conditions (Du Clos & Mold, 2001). The protein has a complex role in both the innate and acquired immune system, by interacting with specific receptors on phagocytic cells, stimulating the production of anti-inflammatory cytokines and activating the classical complement pathway. Furthermore, it may inhibit fatal side effects of bacterial substances by protecting against inflammatory response evoked by lipopolysaccharide (LPS) and cytokines (Du Clos & Mold, 2001). It has also been reported that CRP prevents the chemotaxis and respiratory burst of neutrophils (Mortensen & Zhong, 2000).

Serum CRP concentrations are generally significantly higher in dogs with pyometra compared to healthy control dogs (Fransson *et al.,* 2003; Hagman *et al.,* 2006; Jitpean *et al.,* 2014b; Karlsson *et al.,* 2013). It has also been reported in some studies that elevated levels of CRP is associated with outcome as measured by length of hospitalization (Fransson *et al.,* 2003; Hagman *et al.,* 2006). In the study by Jitpean *et al.* (2014b) the CRP levels in the bitches that stayed longer in the animal hospital after surgical treatment did however not differ significantly from the levels in the bitches with normal postoperative hospitalization. This difference between studies may depend on a low number of dogs included, or the selections of bitches studied.

Concentrations of serum CRP were significantly higher in dogs diagnosed with nonseptic SIRS or sepsis, than in healthy control dogs in a study by Gebhardt *et al.* (2009) with objective to measure serum concentrations of CRP in dogs diagnosed with nonseptic SIRS or sepsis over a three-day-period. When comparing the dogs with nonseptic SIRS to the dogs with sepsis, it was concluded that CRP concentrations did not differ significantly between these groups. The non-surviving dogs with nonseptic SIRS or sepsis did not display a substantial decrease in CRP, which surviving dogs did, suggesting it may be possible to make assumptions regarding the odds of survival. However, it was shown that CRP alone could not be used as a satisfactory

parameter for assessment of survival odds, and that using CRP solely as the basis for decisions regarding therapy or euthanasia is not reasonable. Although, careful interpretation of CRP levels when measured in serial changes of the concentrations in addition to other clinical examinations and laboratory parameters was considered might be beneficial when evaluating the severity of the disease.

MATERIAL AND METHODS

Study design and inclusion criteria

This study was designed as a prospective clinical study. Dogs diagnosed with naturally occurring pyometra and treated surgically with OHE at KKU Animal Hospital, Khon Kaen, Thailand, from August 2016 to October 2016 were eligible to be enrolled into the study upon the owner's written consent. The inclusion criteria comprised a tentative diagnosis of pyometra (made based on history, clinical signs, ultrasonographic findings, and hematological and biochemistry parameters) and positive bacterial growth from the uterine swab. The exclusion criteria comprised bitches younger than 7 months, and bitches showing aggressive behaviour during handling. Additionally, 15 purebred and crossbred clinically healthy bitches admitted to KKU for elective spaying (ovariohysterectomy) were enrolled as control group. The final diagnosis of pyometra was confirmed by histopathological examination of the uterus, performed by F. Södersten at the Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden.

Clinical examination and SIRS-criteria

A complete physical examination including assessment of body weight, heart and respiratory rates, body temperature, hydration status, mucous membrane colour, capillary refill time, location of pain response during abdominal palpation and altered mentation were performed in all dogs on admission to the clinic by personnel in the Surgical department, KKU Animal Hospital, Khon Kaen, Thailand. A similar clinical examination was performed pre-surgery exclusively for the study, with the same veterinarian performing the examination on each dog enrolled in the study to minimize the source of error due to subjective measurement. SIRS-status determination was performed in all dogs and defined as positive if $\geq$ 2 of the following criteria was met: abnormal body temperature (>102.6°F [39.2°C] or <100.6°F [38.1°C]), elevated respiratory rate (>20 breaths/min), elevated heart rate (>120 beats/min), and abnormal number of WBC (>16.000/μL or <6000/μL), or >3% band neutrophils (Hauptman *et al.*, 1997). Additionally, history was noted about each dog, including when the patient last was in heat, hormonal treatment, whelping history and if the patient had any co-morbidity.

Ethical approval

The study was approved by the Animal Ethics Committee of Khon Kaen University on the 21th of July 2016 (record No. ACUC-KKU-43/2559, reference No. 0514 1 75/49).

Hospitalization

In general, bitches treated surgically with OHE due to pyometra at KKU Animal Hospital are hospitalized 1-2 days. Prolonged hospitalization (defined as ≥ 3 days) is only warranted when complications occur or when the general condition of the bitch is depressed, requiring additional veterinary care and monitoring.

Blood, culture and tissue samples

Blood samples

All dogs enrolled in the study (both pyometra and controls) were sampled before surgical treatment (OHE), 24 h ± 2 hours post-operatively and 7-10 days post-operatively when the dogs returned to the clinic for suture removal. Blood samples was collected by venipuncture of the distal cephalic vein, or the saphenous vein and directly transferred into Citrate, nonadditive and EDTA collection tubes (Vacutainer, Becton-Dickinson, Stockholm, Sweden), respectively. The tubes were centrifuged at 3000 rpm for 10 min within 30 min of sample collection, allowing the sample in the nonadditive tube to clot in room temperature for a minimum of 20 min. The serum and plasma from each tube were immediately transferred into 2 mL cryogenic vials (Nunc CryoTubes, VWR international, Stockholm, Sweden) and freeze-stored at approximately -20°C until transportation to Sweden by World Courier with a temperature control service ensuring that all samples were in -20°C during transportation. The samples arrived at SLU >24 hours after departure from KKU. The tubes were coded so that the identity of the sample was only known by the investigator and not the laboratory. The analysis of albumin, iron and CRP were performed within 3 months of sample collection.

Blood cultures

In the dogs with pyometra, blood samples for bacterial cultures were aseptically collected in a sterile syringe before surgical treatment (OHE) and 2 mL of blood was added to a blood culture bottle (Clignac Co Ltd, Bangkok, Thailand). The hemocultures were handled by personnel at the Veterinary Clinical Microbiological Laboratory, KKU Animal Hospital, where they were kept in culturing bottles containing medium and 2 ml blood overnight, and then moved to blood agar for 24 h (Hemoculture: Blood agar, Oxoid Microbiology products, Oxoid limited, UK). Cultures were then moved to MacConkey agar and Chocolate agar (aerobic bacteria) and Thioglycollate broth (anaerobic bacteria) and cultured for a maximum of 5 days. Species were determined using RapID systems (Remel inc., Santa Fe, USA). To determine sensitivity, the bacteria were cultured in Brain heart infusion broth and incubated at 35-37°C for 4-6 h, centrifuged and then transferred into Mueller-Hilton agar. Antimicrobial sensitivity tests were interpreted by using the modified Kirby-Bauer method (Boyle *et al.*, 1973).

Uterine cultures and tissue samples

In the dogs with pyometra, a bacterial sample of the endometrium and contents of the uterus was performed immediately after the uterus had been removed during surgery (OHE) using a sterile cotton swab. The swab was inserted into the uterus via a stab incision of a sterile scalpel. The swab was put in Stuart's transport medium and analysed concerning bacterial species and

drug sensitivity at the Veterinary Clinical Microbiological Laboratory, KKU Animal Hospital, according to the same procedure as described above. Three tissue samples were also collected from the uterus, one from each uterine horn and one from corpus uteri. The tissue samples were approximately 1×1 cm pieces of the entire uterine wall, removed aseptically from the uterus with a sterile scalpel and anatomic forceps. The tissue samples were fixated in 10 % formaldehyde solution in separate containers for a minimum of 3 days, and thereafter embedded in paraffin by personnel at Veterinary Clinical Pathology Laboratory, KKU Animal Hospital. Embedded in paraffin, the tissue samples were transported to Sweden by World Courier for histopathological examination performed at Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden.

Laboratory analysis

Hematology and biochemistry

Standard blood analyses performed at KKU Animal Hospital were included in the study. No additional analyses were performed exclusively for the purpose of the study. Routinely performed hematological analyses consisted of total white blood cell count (WBC) including differential counts, hemoglobin (HB), packed cell volume (PCV), red blood cell count (RBC) and platelet count. The hemathology were analysed by using ABXMicros ES 60 (Horiba medical, Irvine California CA, USA) and differential white blood cell count were performed microscopically. Standard biochemical analysis included creatinine and alaninaminotransferas (ALT). The serum biochemistry parameters were analysed by using Olympus AU 400 (automatic chemistry analyser, International Co., Ltd, Bangkok, Thailand) and Reflotron® (Boehring Mannheim, Germany). Additionally, routine microscopic examination of blood smear for blood parasites were performed in each patient, as tick-borne diseases such as *Ehrlichia canis, Hepatozoon canis, Anaplasma canis* and *Babesia* are commonly found in dogs in the geographical area. In some dogs SNAP-test for *Anaplasma canis* and *Erlichia canis* were also used. All analyses were performed by personnel at the Veterinary Diagnostic Laboratory, KKU Animal Hospital, Khon Kaen, Thailand.

Inflammatory parameters

C-reactive protein was analysed by using Architect C4000, Abbott Diagnostics. Antibodies are utilized to react against CRP in serum, and the immunoaggregation is measured by optic means. The method is specifically adapted to dogs, i.e. the antibodies are directed against canine-CRP (Hillström *et al.*, 2014). The detection limit of the method is 0-300 mg/L, and the reference range for healthy dogs <7 mg/L. Measurement of albumin concentrations was performed by using Architect C4000, Abbott Diagnostics. Albumin BCG is based on specific binding of bromocresol green to albumin, resulting in a coloured complex. The absorbance of the complex at 628 nm is in direct proportion to the concentration of albumin in the sample. Detection limit of the method is 4-105 g/L, and the reference range for healthy dogs is 27-37 g/L. Iron concentrations were analysed using a direct colorimetric determination (Abbott Laboratories Inc., IL, USA) with detection limit 0.9 µmol/L. The reference range of iron in healthy dogs is 14-48 µmol/L. All analyses were performed by trained personnel at the Clinical Chemisty Laboratory, SLU, Uppsala, Sweden.

Statistical analysis

The program Minitab EXPRESS version 1.3.0 for Mac OS X (Minitab Inc. State College, PA, USA) was used for statistical analyses. 2-sample t-test and ANOVA were used for normally distributed variables. The significance level was set to $P < 0.05$ for all tests in the study.

RESULTS

Initially, 10 purebred or crossbred bitches with the preliminary diagnosis pyometra were included in the study. One bitch (P04) was thereafter excluded (despite an enlarged uterus detected during ultrasonographic examination and during OHE) due to a negative uterine bacterial culture (no growth after 48 h) and histopathological findings not indicative of pyometra. The final pyometra group comprised of 9 bitches. In the pyometra group, the mean ± SD age was 7.4 ± 4.1 years, with a range of 10 months to 15 years. In the control group, the mean ± SD age was 1.9 ± 1.7 years, with a range of 7 months to 10 years. The age differed significantly between the two groups (higher in dogs with pyometra) ($p = 0.004$). The mean ± SD body weight for the pyometra group was 20.5 ± 8.3 kg (range 4.3-28.5 kg) and in the control group 15.1 ± 6.9 kg (range 6.9-33.8 kg). The body weight did not differ significantly between the two groups ($p = 0.127$). The pyometra group included 6 nulliparous, 2 primiparous, and 2 pluriparous bitches, and the healthy control group included 10 nulliparous, 2 primiparous, and 3 pluriparous bitches.

Albumin concentrations were significantly lower in the pyometra group compared to the control group ($p = 0.0001$). In contrast, CRP concentrations were significantly higher in the pyometra group compared to the control group ($p = 0.0003$). Iron concentrations did not differ significantly between the two groups ($p = 0.483$) (Table 1).

When comparing the different sample occasions (pre-operatively, 24 h post-operatively and 7-10 days after surgery) in the pyometra group, all bitches had lower concentrations of albumin 24 h post-operatively than before surgery. The concentrations of CRP were highest pre-operatively in the bitches with pyometra, and had decreased (although not significantly) 24 h post operatively. In the control bitches the concentrations of CRP differed significantly ($p = <0.0001$) when comparing the pre-operatively sample to the post-operatively sample (higher CRP concentrations post-operatively). In both the pyometra and the control groups, CRP levels had decreased significantly in the sample collected at suture removal compared to the post-operative sample i.e. the CRP concentrations were lower at suture removal ($p = 0.0004$, and $p = <0.0001$, respectively).

Table 1. *Mean ± standard error of the mean (SEM) concentrations of CRP, iron and albumin measured in bitches with pyometra and healthy control dogs.*

Variable	Mean (±SEM)		Reference range[a]
	Pyometra group (range)	Control group (range)	
Albumin (g/L)	19.4 ± 1.5 (14.3-28.6)	29.1 ± 1.2 (19.7-34.8)	27-37
Iron (μmol/L)	20.1 ± 2.9 (9.0-36.1)	22.8 ± 2.3 (6.3-38.6)	14-48
CRP (mg/L)	162.9 ± 24.4 (74.6-299.3)	11.7 ± 1.9 (5.9-30.7)	<7

Abbreviations: CRP, C-reactive protein

[a] Reference range at the Clinical Chemisty Laboratory, University Animal Hospital SLU, Uppsala, Sweden.

In 3 of the 9 bitches (33 %) with pyometra, the postoperative hospitalization was prolonged (≥3 days). There were too few bitches in the pyometra group to investigate statistical association between concentrations of albumin, iron and CRP and prolonged hospitalization, but no obvious deviant concentrations of these inflammatory markers were noted in the bitches with prolonged hospitalization. One of the bitches with pyometra died the night after surgery, resulting in a mortality rate of 11 %.

Six of the nine bitches with pyometra were SIRS positive (SIRS+) (Table 2). Two of the SIRS+ bitches (33%) had a positive blood culture (Table 3). There were too few bitches in the pyometra group to investigate statistical association between concentrations of albumin, iron and CRP and SIRS-status, but no obvious deviant concentrations of these inflammatory markers were noted in the SIRS+ bitches. The results of the bacterial culturing from the uterus in bitches with pyometra are shown in Table 3.

When examined histologically, the uterine tissue samples from 8 dogs showed signs of pyometra (neutrophils and debris in lumen). In 6 of these dogs cystic endometrial hyperplasia (CEH) was also present. The tissue samples from 4 dogs displayed purulent endometritis, and one dog had a mild lymphoplasmacytic endometritis. In the samples from one dog (P08) no signs of pyometra could be detected, despite a positive uterine bacterial culture.

Table 2. *Clinical examination parameters and SIRS-status in bitches with pyometra*

ID	Heart rate (beats/min)	Respiratory rate (breaths/min)	Body temperature (°C)	WBC/µL	SIRS +/-[a]
P01	120	Panting	38.9	12.700	-
P02	136	64	38.9	55.400	+
P03	124	100	38.6	18.800	+
P05	104	34	38.9	12.000	-
P06	84	40	37.8	51.300	+
P07	124	80	40.0	16.800	+
P08	120	16	38.3	10.600	-
P09	132	36	37.8	30.900	+
P10	128	36	37.7	71.000	+

[a] SIRS status defined by criteria validated by Hauptman *et al.* (1997)

The results of hemathology and serum biochemistry analyses are given in Table 4. The WBC and creatinine levels differed significantly between the bitches with pyometra and the control group (higher in bitches with pyometra) ($p = 0.032$ and $p = 0.035$, respectively). The morphology of the neutrophil granulocytes was examined in all blood samples. Three of the cases (33 %) showed hyposegmentation of the neutrophils, i.e. band neutrophils, and one of the cases (11 %) showed toxic granulation of the neutrophils. All of these bitches were SIRS+ and had a uterine infection with Gram-negative bacteria.

Table 3. Bacterial isolated from the uteri of the bitches with pyometra

ID	Uterine swab (growth)	Blood culture (growth)
P01	*Streptococcus sp.* (non *S. agalactie*) (N[a]) *Proteus mirabilis* (M[b])	Neg[d]
P02	*Klebsiella oxytoca* (N[a])	Neg[d]
P03	*Escherichia coli* (N[a])	*Escherichia coli* (N[a])
P05	*β-hemolytic Escherichia coli* (N[a])	Neg[d]
P06	*Escherichia coli* (N[a])	Neg[d]
P07	*Escherichia coli* (N[a])	Neg[d]
P08	G+[c] *Bacilli* (M[b])	Neg[d]
P09	*Staphylococcus intermedius* (N[a]) *β-hemolytic Escherichia coli* (N[a])	*Staphylococcus intermedius* (N[a])
P10	*Escherichia coli* (N[a])	Neg[d]

[a] Numerous growth, [b] Moderate growth, [c] Gram-positive, [d] No growth after 5 days

Table 4. *Hematology and serum biochemistry*

Variable	Mean (±SEM[b])		Reference range[a]
	Pyometra group (range)	Control group (range)	
WBC[c] (x10^3/mm^3)	31 ± 7.5 (10.6-71)	11.4 ± 7.5 (6.1-17.2)	6-15
PCV[d] (%)	29 ± 3.5 (16-44)	47.3 ± 3.5 (32-66)	38-55
Hemoglobin (g/L)	10.2 ± 1.2 (5.6-15,3)	15.3 ± 0.6 (11.1-19.6)	12-18
RBC[e] (10^6/mm^3)	4.4 ± 0.5 (2.14-6)	6.7 ± 0.3 (4.6-8.05)	6-9
Neutrophils (%)	78.4 ± 3.2 (64-89)	6.7 ± 2.5 (46-78)	60-75
Band neutrophils (%)	0.1 ± 0.1 (0.0-1.0)	0.07 ± 0.07 (0.0-1.0)	1-4
Lymphocytes (%)	13.3 ± 3 (5-34)	24.5 ± 2.3 (9-38)	12-30
Monocytes (%)	7.2 ± 1.8 (0-18)	3.9 ± 1 (0.0-11	3-9
Eosinophils (%)	1.1 ± 0.5 (0-5)	8.6 ± 1.4 (1-20)	2-10
Basophils (%)	0.0 (0.0)	0.07 ± 0.07 (0.0-1.0)	0-1
Creatinine (mg%)	1.8 ± 0.4 (0.57-3.65)	0.78 ± 0.05 (0.3-1.06)	0,5-1,8
ALT[f] (U/L)	27.9 ± 7.8 (10-85)	48.4 ± 7 (19-101)	10-125

[a] Reference range at the Veterinary Diagnostic Laboratory, Khon Kaen University Animal Hospital, Thailand, [b] Standard error of the mean, [c] Total white blood cell count, [d] Packed cell volume, [e] Red blood cell count, [f] Alkaline phosphatase

DISCUSSION

The results from this study showed that concentrations of serum albumin are lower in bitches with pyometra than in healthy bitches. This is in agreement with the result of a previous study performed in Sweden (Hagman, 2011a) and as expected for this negative acute phase protein as the disease likely is similar regarding the inflammatory changes independently of country. The fact that all bitches with pyometra had lower concentrations of albumin 24 h post-operatively than before surgery might be explained by the tissue damage and the inflammatory response caused by surgery (Desborough, 2000). However, according to this theory, it would be expected to find lower concentrations of albumin 24 h post-operatively also in the control dogs, which was not the case. Several healthy dogs had in fact slightly higher concentrations of albumin the day after surgery, suggesting that the trauma caused by OHE is not sufficient to cause an inflammatory response strong enough to result in decreased concentrations of albumin in healthy dogs. These results imply that bitches already circulatory affected due to pyometra (the inflammatory response causes increased capillary vascular permeability and development of hypotension) have a more persistent hypoalbuminaemia compared to the healthy control bitches that are solely exposed to the surgical trauma. The final blood sample was taken when the bitches returned to the clinic for suture removal, and in all bitches with pyometra albumin levels were increased although none of the samples were within the range for healthy dogs. There was no significant difference in iron concentrations pre-operatively when comparing the pyometra group with the control group. This is not in agreement with the previous findings of lower concentrations of iron in bitches with pyometra (Jitpean *et al.,* 2014c) and decreased iron concentrations in response to infection (Cartwright *et al.,* 1946).

C-reactive protein concentrations were significantly increased in bitches with pyometra pre-operatively compared to that in the healthy dogs, which is in agreement with reports from several previous studies (Fransson *et al.,* 2003; Hagman *et al.,* 2006; Jitpean *et al.,* 2014c; Karlsson *et al.,* 2013). Interestingly, only 3 of the 15 (20 %) control dogs included in the study had CRP concentrations within the reference range of healthy dogs (<7 mg/L) before surgery despite that all dogs were determined to be clinically healthy based on physical exam and the case history. This discrepancy could be explained by the different way of housing dogs in Thailand compared to Sweden, since most dogs were kept outside at all times, often roaming freely, as compared to being kept indoors and as family members in Sweden. This manner of keeping the dogs in Thailand resulted in less meticulous control of the dogs, including dietary factors. This in combination with a different food culture, where food where commonly sold in small market stalls along the streets, promotes a likelihood that many dogs found and ate left-overs, which possibly could result in a low grade inflammatory response. Additionally, since there were a large number of unattended stray dogs in the study population, there was a general problem with ectoparasites that could have influenced the results of laboratory and clinical parameters investigated. Previously, longer duration of hospitalization and presence of sepsis in bitches with pyometra has been shown to be associated with higher CRP concentrations (Fransson *et al.,* 2007). In the present study, CRP concentrations in SIRS-positive bitches and/or bitches in the bitches that stayed longer in the animal hospital after OHE did not differ significantly from the bitches that had regular postoperative hospitalization. This difference

between studies could be because of the relatively low number of dogs included in the present study and the selection of bitches studied.

In this study, anaemia and leukocytosis were common findings in bitches with pyometra, which in agreement with the results of most studies of the disease (Fransson *et al.*, 1997; Jitpean *et al.*, 2014b; Maretta *et al.*, 1989). The bitch that died the night after surgery had remarkably high WBC ($71x10^9$/L) compared to the other bitches with pyometra (range 10,6-$55,4x10^9$/L). The physical examination findings in this bitch also deviated from the other bitches with pyometra in that the bitch was severely depressed at admission (not being able to stand up and was brought in on a stretcher lying flat on it's side). This particular bitch also had peritonitis, which was discovered during surgery. Peritonitis has been reported to be the most common complication in dogs with pyometra and it can be life-threatening (Jitpean *et al.*, 2014b). In two other pyometra dogs in the study peritonitis was discovered during surgery. All the three dogs with peritonitis were SIRS-positive, and one of them also had a positive blood culture confirming bacteremia. One other bitch had a positive blood culture, but the bacterium isolated (*Staphylococcus intermedius*) is part of the normal skin and oral flora in dogs, this result could also reflect contamination. However, the same bacterium was also isolated from the uterus of this bitch, which indicate that the result indeed is valid, due to the lesser likelihood of two samples (taken on different occasions using different methods) both being due to contamination.

The most common bacterium isolated from the uteri of the bitches with pyometra was *E. coli*, which is in accordance with other reports (Fransson *et al.*, 1997; Bigliardi *et al.*, 2004; Dow, 1959; Sandholm *et al.*, 1975). It has been shown that *E. coli* has an affinity for progesterone-primed endometrium (Sandholm *et al.*, 1975), which could explain this predominance. One bitch had an infection solely with a Gram-positive bacterium (G^+ *Bacillus sp.*), and when examined histologically the tissue samples from this bitch did not show any signs of pyometra. Despite the result from the histological examination, the bitch was included in the study since all inclusion criteria were fulfilled (history, clinical signs, ultrasonographic findings, and hematological and biochemistry parameters conforming with the diagnosis of pyometra, and positive growth from the uterine bacterial swab). The macroscopic examination was not included in the inclusion criteria, for practical reasons (large uteri collected in Thailand and needing transportation), thus making it difficult to assess the presence of pus in lumen of the uterus, due to the fact that much of it (sometimes all of it) can disappear while handling the tissue samples (or at paraffin embedding).

Three of the eight *E. coli* isolates from pyometra uteri were resistant to more than one antimicrobial drug. All of these isolates were resistant to third generation fluoroquinolone (marbofloxacin and enrofloxacin), two of them were resistant to tetracycline, and one was resistant to trimethoprim-sulfametoxzol. Resistance to three or more antimicrobials (multiresistance) could not be observed in any of the isolates. However, the interpretation of the antimicrobial susceptibility tests was complicated by the fact that there was no standard profile of the antimicrobials tested. Instead, the veterinarian in charge chose which antimicrobials that were to be included in the antimicrobial sensitivity profile for each sample. Since different veterinarians handled the samples from case to case, the profile tested was not

the same for each bitch and were thus not directly comparable. For example, ampicillin was only tested for six of the thirteen isolates, which makes it impossible to draw any conclusions regarding resistance to this antibiotic drug. This would otherwise have been interesting, since resistance to ampicillin has been the most commonly observed trait among isolates of *E. coli* from bitches with pyometra in Sweden (Hagman & Greko, 2013). However, the routinely distributed of antimicrobials at KKU Animal Hospital, Thailand, differ from the current standard routines at UDS, SLU, Sweden (2016). The most commonly used antimicrobial drug in the bitches with pyometra as well as in the healthy bitches admitted for elective spaying at KKU Animal Hospital, Thailand, is first generation cephalosporines (Cefazolin and/or Cephalexin) and were distributed to 23 of the 24 dogs included in the study (96 %). Other antimicrobial drugs prescribed to the bitches with pyometra were enrofloxacin (n = 2), metronidazole (n = 4), doxycycline (n = 2) and amoxicillin (n = 1). One of the control dogs also received enrofloxacin due to wound infection at time for suture removal. All dogs included in the study (both pyometra and control dogs) were treated with antimicrobial drugs for a minimum of 8 days. In comparison, post-operative antimicrobial treatment is solely used post-operatively in systemically ill bitches or bitches with signs of or high risk of developing complications at UDS, SLU, Sweden.

The routine administration of broad-spectrum antibiotics post-operatively at KKU Animal Hospital could be due to a combination of factors; the warm and moist climate in Thailand is more optimal for bacterial growth, the dogs are generally kept outside by the owners resulting in a greater risk for contamination of the wound post-operatively, and the hygiene routines at the surgical ward differs from those at UDS, SLU, Sweden. In KKU Animal Hospital, very little disposable material was used (surgical drapes and gowns were made of cloth and washed and re-used), jodopax-solution was often used to sterilize surgical instruments, and hygiene zones between the operating theatres and preparation room was basically non-existent. Of these factors, it is likely the general way the dogs are being held by the owners in combination with the warm climate having the most impact of the routinely distribution of broad-spectrum antibiotics in order to prevent complications in form of, for example, wound infection. By being held outside, roaming around and coming in contact with other dogs and animals, the exposure of a various bacterial flora is likely greater for the bitches surgically treated for pyometra in Khon Kaen, Thailand compared to those treated in Sweden. Also the warm climate composes a risk that bacteria contaminating the wound are given optimal conditions for colonisation and growth. The use of an Elizabethan collar postoperatively was not practised, neither in the clinic nor when the bitches were sent home, thus allowing the dogs to lick the surgical wound. Additionally, the bitches admitted for pyometra were often severely ill at admission, because financial difficulties were a common situation among the owners. This financial situation resulted in the owners generally waiting with seeking veterinary care until the bitches were in very poor clinical condition, which was reflected in that most bitches with pyometra included in the study had shown signs of illness for 2-3 days before admission to the clinic, but some had been ill 1-2 weeks at the time for admission. The financial restriction is also a possible explanation to the routinely administration of antibiotics, since revisits at the clinic and further costs due to veterinary care for treatment of complications (such as wound infection) comprised an additional cost that many owners could not afford. It was suboptimal to use length of

hospitalization in the present study as a measurement of morbidity, as it depended more on financial situation of the owners and less on the severity of the dog's illness and most dogs were dismissed the same day or the day after surgery. However, hospitalization of 3 days or more was determined as prolonged in this study, after discussion with the Head veterinarian of the surgical ward regarding the general hospitalization when financially possible.

One other noticeable and important difference between Sweden and Thailand was that euthanasia is not practised as a rule in Thailand due to cultural and religious reasons. This became evident in one of the bitches surgically treated for pyometra, which is addition to being 15 years old and diagnosed with pyometra was suffering from severe periodontitis, cataract of both eyes and had a moderate degree of heart murmur. Still, euthanasia was not considered an option for this bitch.

There were several limitations of this study. The number of bitches with pyometra admitted to KKU Animal Hospital during the sample collection period was lower than anticipated. This resulted in relatively few dogs included, and limited possibly for reaching enough power in the statistical analyses. Because of the language barrier, all communication with the owners was dependent on the veterinarian in charge, resulting in translated answers regarding all case history questions and the other information about the bitches. The aim to collect samples for blood culturing before the dogs had received any antibiotic treatment proved to be very difficult, since antibiotics generally were administered in an early stage of the treatment, either since the dogs suffered from tick-borne diseases (*Ehrlichia canis, hepatozoon canis* and *Babesia*) or while they were waiting for surgery (OHE due to pyometra was only performed during daytime on weekdays). At the time of the clinical examination performed pre-surgery due to the study, most dogs had already received treatment with antibiotics and fluid therapy, which is likely to affect the results of the physical examination. The physical examination was often performed without the owner and in an environment with other dogs present. This likely resulted in a stress response, which in turn could induce falsely high heart- and respiratory rates and body temperature in the dogs examined. The warm and humid climate also comprised a difficulty, since most dogs were panting during the examination, making it impossible to count the respiratory rate. These factors made decision of SIRS-status based on all criteria validated by Hauptman *et al.* (1997) somewhat uncertain, which further pinpoints the need to find suitable objective and clinically usable biomarkers in order to achieve early and accurate diagnosis of sepsis in dogs.

CONCLUSIONS

The inflammatory response induced by pyometra resulted in decreased systemic concentrations of albumin, and increased systemic concentrations of CRP. Iron concentrations did not differ significantly between bitches with pyometra and healthy control dogs. None of the investigated inflammatory markers did differ between septic and non-septic bitches, which indicate that none of the investigated parameters have a potential clinical value for detection of sepsis. However, a larger study material would be useful for further evaluation. Broad-spectrum antibiotics are used routinely in bitches treated surgically for pyometra and bitches admitted for elective spaying at KKU Animal Hospital. The most commonly used preparation was first-generation cephalosporines (Cefazolin and/or Cephalexin) and the general length of treatment

was 8 days. Multiresistance could not be observed in any of the isolates from the uteri bacterial swabs, but a larger study material and a standard profile of antimicrobials tested would be beneficial to give a more correct interpretation of the antimicrobial susceptibility tests and to survey antibiotic resistance.

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