

New Aspects of Canine Pyometra

Studies on Epidemiology and Pathogenesis

R. Hagman

*Department of Small Animal Clinical Sciences
Uppsala*

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To my Family

Abstract

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Pyometra is a common and lethal disease in bitches characterised by uterine bacterial infection leading to subsequent systemic illness. The objectives of the present thesis were to investigate the incidence of the disease in relation to breed and age, to assess bacteriological aspects of pyometra and to evaluate the involvement of endotoxin and prostaglandin F_{2α} in the pathogenesis.

Animal insurance data revealed age- and breed-dependent differences in the incidence of pyometra. On average 23-24% of all bitches studied experienced pyometra before 10 years of age. Data presented in this study indicate that certain breeds have a genetic predisposition for pyometra.

Bacteriological genotype examinations showed that pyometra is most likely caused by *Escherichia coli* clones originating from the normal flora of each dog, i.e. not by clones spreading between animals. The resistance among *E. coli* isolates from pyometra bitches against antimicrobials commonly used in canine practice was low and not likely to cause therapy failure. Data on antimicrobial resistance of *E. coli* from urinary tract infections were generally not suitable for selecting antimicrobial treatment of pyometra.

Systemic endotoxemia was confirmed in bitches with pyometra. The plasma levels of endotoxin were correlated with concentrations of the prostaglandin F_{2α} metabolite (PG-metabolite). This indicates the usefulness of PG-metabolite in the diagnosis of endotoxemia in bitches. Bitches with pyometra also had increased blood concentrations of PG-metabolite compared with bitches with cystic endometrial hyperplasia (CEH). The present study revealed that in bitches with fluid in the uterus, the analysis of PG-metabolite in combination with percentage band neutrophils can distinguish between pyometra and CEH. The levels of PG-metabolite are predictive of the severity of pyometra since they were correlated to criteria of a systemic inflammatory response and also to the length of hospitalisation.

In summary, this thesis provides data on breed- and age-related differences in the incidence of pyometra, which will be helpful in future studies of the disease and breeding programmes. In addition, clarification of key bacteriological and pathophysiological characteristics of the development of pyometra can improve diagnostic and therapeutic strategies and increase survival rates.

Keywords: Dogs, urinary tract infection, pulsed-field gel electrophoresis, lipopolysaccharide, limulus amoebocyte lysate assay, minimum inhibitory concentration, antibiotics.

Author's address: Ragnvi Hagman, Department of Small Animal Clinical Sciences, Swedish University of Agricultural Sciences, Box 7037, SE-750 07 UPPSALA, Sweden.
Ragnvi.Hagman@kirmed.slu.se

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Abbreviations

ALAT	Alanine aminotransferase
Alb	Albumin
A/G	Albumin - globulin quote
ALP	Alkaline phosphatase
ASAT	Aspartate aminotransferase
BA	Bile acids
BUN	Blood urea nitrogen
Urea	Blood urea nitrogen
BN	Band pneutrophils
CEH	Cystic endometrial hyperplasia
CHEF	Contour-clamped homogenous electric field
CK	Creatine kinase
CNF	Cytotoxic necrotising factor
Crea	Creatine
EPC	Erythrocyte particle concentration
EVF	Erythrocyte volume fraction
ET	Endotoxin
Glu	Glucose
Hb	Hemoglobin
Hct	Hematocrit
HR	Heart rate
LAL	Limulus amoebocyte lysate
MIC	Minimum inhibitory concentration
Mono	Monocytes
PBN	Percentage band neutrophils
PCV	Packed red blood cell volume
PFGE	Pulsed-field gel electrophoresis
PG	Prostaglandin
PG-metabolite	Prostaglandin 15-keto-13,14-dihydro-PGF _{2α} metabolite
Prot	Protein
RIA	Radioimmunoassay
RR	Respiratory rate
SIRS	Systemic inflammatory response syndrome
SLU	Swedish University of Agricultural Sciences, Uppsala, Sweden
SVA	National Veterinary Institute, Uppsala, Sweden
Temp	Body temperature
UTI	Urinary tract infection
WBC	White blood cell count

Appendices

Papers I-V

This thesis is based on the following papers, which will be referred to by their roman numerals:

I. Egenvall, A., Hagman, R., Bonnett, B.N., Hedhammar, Å., Olsson, P. and Lagerstedt, A.-S. 2001. Breed risk of pyometra in insured dogs in Sweden. *Journal of Veterinary Internal Medicine* 15, 530-538.

II. Hagman, R. and Kühn, I. 2002. *Escherichia coli* strains isolated from the uterus and urinary bladder of bitches suffering from pyometra: comparison by restriction enzyme digestion and pulsed-field gel electrophoresis. *Veterinary Microbiology* 84, 143-153.

III. Hagman, R. and Greko, C. 2004. Antimicrobial resistance in *Escherichia coli* from canine pyometra and urinary tract infections. *The Veterinary Record* (In press).

IV. Hagman, R., Kindahl, H. and Lagerstedt, A.-S. 2004. Pyometra in bitches induces elevated plasma endotoxin and prostaglandin F_{2α} metabolite levels. (Manuscript submitted for publication).

V. Hagman, R., Kindahl, H., Fransson, B.A., Bergström, A., Ström Holst, B. and Lagerstedt, A.-S. 2004. Differentiation between pyometra and cystic endometrial hyperplasia in bitches by prostaglandin F_{2α} metabolite analysis. (Manuscript submitted for publication).

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Introduction

Pyometra in bitches - a background

Pyometra is a common metoestral disease of intact adult bitches (Dow, 1958). It is characterised by uterine bacterial infection with pus accumulating in the uterus and systemic illness (Børresen, 1975). The disease is associated with a variety of clinical symptoms and is life-threatening in severe cases (Sevelius, Tidholm & Thorén-Tolling, 1990; Okano, Tagawa & Takase, 1998). Despite being an important reproductive disease and the focus of research in canine veterinary medicine since the 1920's (Möller Sørensen, 1929), central aspects of the complex aetiology and pathogenesis of the disease are obscure or unknown.

Several terms, such as chronic endometritis, chronic purulent metritis or cystic endometrial hyperplasia - pyometra complex, have been used in the literature to describe the condition (Teunissen, 1952; Dow, 1957; Sandholm, Vasenius & Kivistö, 1975). The definition of canine pyometra as a chronic purulent endometritis post estrum, with or without polysystemic effects will be used throughout this thesis (adapted after Hardy & Osborne, 1974).

In Scandinavian countries, a large dog population is at risk of developing uterine disorders such as pyometra since elective neutering of healthy bitches is seldom performed. In Sweden neutering (spaying) is mainly performed as a result of medical problems and consequently only 7% of the bitches are neutered (Egenvall *et al.*, 1999). The situation is different in many other countries, e.g. in the USA (Manning & Rowan 1992) and Australia (Blackshaw & Day, 1994) where elective spaying is common practice and performed in 85% and more than 50% of all bitches, respectively.

The possible predisposition for pyometra in different breeds has been evaluated in a few studies, but was not assessed in relation to the base dog population (Krook, Larsson & Rooney, 1960; Ewald, 1961; Niskanen & Thrusfield, 1998). Increased risk of the development of pyometra has been associated with e.g. hormone therapy and nulliparity (Niskanen & Thrusfield, 1998), whereas for contrast overt pseudopregnancy might be a protective factor (Fidler *et al.*, 1966).

Pathogenesis

Pyometra is developed as a result of a complex of aetiological factors. These include the hormonal influence on the uterine environment, virulence of the infecting bacteria, the general ability of the bitch to combat the infection and the individual sensitivity to bacterial and inflammatory products (Fig. 1).

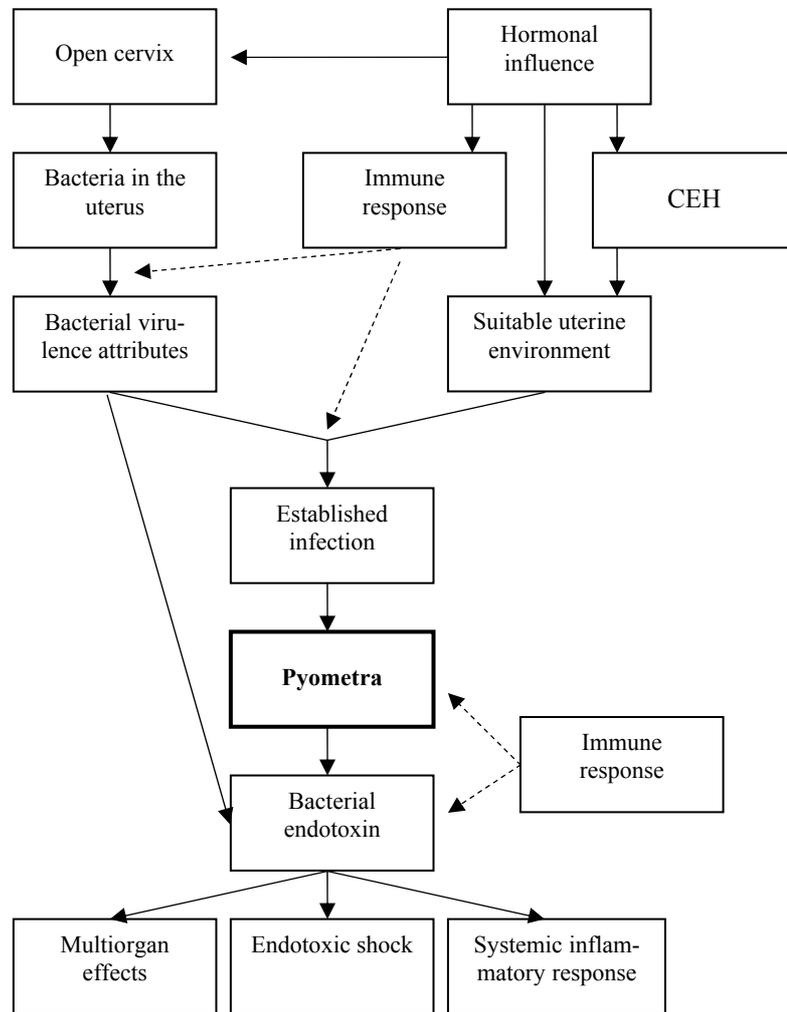


Fig. 1. Conceptual and generalised model of factors involved in the pathogenesis of canine pyometra.

The CEH-pyometra complex

It is generally believed that cystic endometrial hyperplasia (CEH) is caused by repeated exposure of the endometrium to progesterone during the relatively long luteal phase of the oestrus cycle in bitches (Hardy & Osborne, 1974). Presence of CEH predisposes the uterus for secondary bacterial infection leading to pyometra (Dow, 1959a, 1959b). In CEH the endometrial walls are thickened with a pathological proliferation and growth of the endometrial glands (Dow, 1958). CEH can be accompanied by sterile fluid in the uterine lumen which is defined as mucometra or hydrometra depending on the degree of hydration of the mucin (Dow, 1958). The act of repeated progestational stimulation in the pathogenesis is supported by the fact that CEH and pyometra mainly affect middle-aged and older bitches (Benesch & Pommer, 1930; Dow, 1958). The concept of the "cystic endometrial hyperplasia - pyometra complex" where CEH initiates a gradually developing pathological process with pyometra as the most severe end-stage, was introduced by Dow (1957). Dow (1957, 1958, 1959a, 1959b) divided bitches with the disease into four groups based on histopathological examinations of the uteri: (I) uncomplicated CEH, (II) CEH with plasmacell infiltration, (III) CEH with acute endometritis and (IV) chronic endometritis/pyometra. However, a recent study suggested that CEH and pyometra should be regarded as two separate entities based on their clinical manifestations and morphohistological differences (De Bosschere *et al.*, 2001). In that study it was also determined that CEH and pyometra can develop independently of each other (De Bosschere *et al.*, 2001).

The role of hormones

Since early attempts to produce pyometra by experimental bacterial infection failed, hormonal dysfunctions were thought to be the most important aetiological factor of pyometra (Benesch & Pommer, 1930; Lesboyries and Berthelon, 1935). Given that pyometra mainly appears in metoestrus and can be produced by experimental progesterone injections, increased or prolonged secretions of progesterone were believed to initiate the disease (Teunissen, 1952; Dow, 1958, 1959a). The progesterone-sensitised uterus is suitable not only for pregnancy but also for bacterial infection since progesterone stimulates endometrial gland growth and secretion, as well as cervical closure and suppression of myometrial contractions (Cox, 1970). In addition, progesterone has been shown to decrease the uterine resistance to bacterial infection in other animal species (Rowson, Lamming & Fry, 1953; Hawk, Turner & Sykes, 1960; Ganjam *et al.*, 1982). Peripheral blood levels of oestrogen and progesterone in bitches with pyometra are, however, not abnormally elevated in most studies (Christie, Bell & Parkes, 1972; Austad, Blom & Børresen, 1979; Hadley, 1975). Contrastingly, elevated blood levels of estradiol-17 β (Ververidis *et al.*, 2004) and progesterone (Vandeplassche, Coryn & De Schepper, 1991) have also been measured in pyometra bitches compared with healthy ones. Oestrogen alone has not experimentally been able to induce pyometra, but it enhances the effect of subsequently administered progesterone (Dow, 1959a; Teunissen, 1952). This could explain why oestrogen therapy has been associated with increased risk of pyometra (Bowen *et al.*, 1985; Dhaliwal, England & Noakes, 1999; Niskanen & Thrusfield, 1998). The synergistic effect of oestrogen and progesterone in the

pathogenesis of uterine disorders is further indicated by the more frequent findings of simultaneous ovarian follicles and corpora lutea in bitches with diseased uteri compared with healthy ones (Ström Holst *et al.*, 2001). Although physiologically present in lower concentrations than in most experimental studies, the functions of the ovary-derived hormones are clearly important in the pathogenesis since bilateral ovariectomy prevents the development of the disease (Lesboyries & Berthelon, 1935).

Recent investigations have explored the possibility of pyometra being induced by an exaggerated uterine response to normal hormone levels (Dhaliwal, England & Noakes, 1997; Ververidis *et al.*, 2004). The expression of steroid receptors has been shown to be different in uteri with CEH and pyometra compared with healthy bitches (De Cock *et al.*, 1997; Sauerwein *et al.*, 1998; De Bosschere *et al.*, 2002; Ververidis *et al.*, 2004). Although yet uncertain, hormone receptors may thus play a role in the pathogenesis of the disease.

The role of bacteria

Bacteria, predominantly *Escherichia coli*, are isolated in most (62-90%) pyometra cases (Grindlay, Renton & Ramsay, 1973; Sandholm, Vasenius & Kivistö, 1975; Fransson *et al.*, 1997; Bigliardi *et al.*, 2004). This predominance might simply be because *E. coli* are natural inhabitants of the vaginal flora and gain entrance to the uterus during proestrus and oestrus (Watts, Wright & Whithear, 1996). The ability of *E. coli* to adhere to specific receptors in progesterone-stimulated endometrium is probably also important for the establishment of infection (Sandholm, Vasenius & Kivistö, 1975). The origin of the *E. coli* strains found in pyometra is most likely the normal flora of the bitch or the urinary tract since a simultaneous subclinical urinary tract infection commonly is present (Sandholm, Vasenius and Kivistö, 1975; Wadås *et al.*, 1996). Normally the healthy uterus is capable of eliminating entering bacteria without further development of uterine pathologies (Watts, Wright & Whithear, 1996). This was experimentally shown by Nomura and co-workers (1988), who inoculated a known pathogenic pyometra-derived *E. coli* strain into the vagina of 216 healthy bitches. Persistent uterine disease was not induced in any of the bitches despite apparent bacterial invasion into the uterine cavities.

Certain serotypes of *E. coli* are isolated more frequently in pyometra (Grindlay, Renton & Ramsay, 1973; Choi & Kawata, 1975; Sandholm, Vasenius & Kivistö, 1975; Dhaliwal, Wray & Noakes, 1998), which most likely depends on virulence factors associated with these serotypes increasing their ability to establish and maintain an infection in the uterus (Chen *et al.*, 2003). One probable virulence trait is production of cytotoxic necrotising factor (CNF), which has been shown to increase endothelial damage and provoke a greater inflammatory reaction in pyometra (Dhaliwal, Wray & Noakes, 1998). The more virulent *E. coli* strains might be spread between dogs, either directly causing disease or be harboured in the normal bacterial flora for longer or shorter times until favourable conditions develop. This has been shown in urinary tract infections in man (Kühn, Tullus & Möllby, 1986).

Clinical manifestations

The clinical manifestations of canine pyometra are well described in the literature (Hardy & Osborne, 1974; Børresen, 1975). A bitch suffering from pyometra is classically presented in metoestrus, with a history of a variety of symptoms associated with both genital and extragenital lesions. A purulent vaginal discharge is often present provided the cervix is open. Common symptoms associated with the polysystemic disease are dehydration, polydipsia, polyuria, lethargy, abdominal pain, anorexia, vomiting or diarrhoea, fever or hypothermia, abnormal colour of the mucous membranes and elevated heart- and respiratory rates (Børresen, 1979). The clinical course of the disease, and consequently the symptoms, vary from prolonged development of chronic uterine inflammation to, more rarely, sudden death in endotoxic shock (Hardy & Osborne, 1974).

Pyometra induces disturbed organ functions which are noted in the hematological and blood biochemical examinations. Classically there is leucocytosis, with neutrophilia and left shift in the differential white blood cell count (Børresen, 1980). Occasionally leucopenia is present (Børresen, 1980). A normocytic, normochromic anemia is thought to reflect the chronicity of the disease, decreased erythropoiesis due to toxic effects on the bone marrow, lack of available iron and loss of erythrocytes to the uterus (De Schepper, Van Der Stock & Capiou, 1987). Dehydration often complicates the evaluation of anemia (Børresen, 1980). Hypoalbuminemia and hyperproteinemia are frequent findings, reflecting loss of albumin via the kidneys and increased production of gammaglobulines (Åsheim, 1965; Børresen, 1980; Børresen & Skrede, 1980). Decreased levels of the enzyme alanine aminotransferase (ALAT), due to inhibition of liver enzyme synthesis or hepatic membrane damage, and increased levels of aspartate aminotransferase (ASAT) are also associated with pyometra (De Schepper, Van Der Stock & Capiou, 1987). Evaluation of admitted bitches with pyometra often includes parameters assessing kidney function since renal disease and renal failure are not uncommon complications to pyometra (Capiou, De Schepper & Van Der Stock, 1987; Stone *et al.*, 1988; Wheaton *et al.*, 1987; Åsheim 1964, 1965). Elevated concentrations of alkaline phosphatase (ALP), bilirubin and cholesterol concentrations probably reflect intrahepatic cholestasis (Børresen, 1980; Børresen & Skrede, 1980; Sevelius, Tidholm & Thorén-Tolling, 1990).

Treatment

The treatment of choice has traditionally been ovariohysterectomy (Hardy & Osborne, 1974), but in some cases the bitches can be too severely affected to survive (Wheaton *et al.*, 1987). Supportive treatments such as intravenous fluids are often administered in conjunction with surgical treatment of pyometra (Johnson, 1995). In Sweden the policy regarding the administration of antimicrobials is restrictive. At the Veterinary Hospital in Uppsala antimicrobial

treatment is only administered to surgically treated pyometra bitches with moderately to severely deteriorated clinical status, peritonitis or surgical complications such as uterine rupture.

Medical treatment of pyometra with compounds promoting expulsion of the uterine pus, in combination with antimicrobials, is an option in some cases depending on the status of uterus and the general condition of the bitch (Meyers-Wallen, Goldschmidt & Flickinger, 1986; Trasch, Wehrend & Bostedt, 2003; Gobello *et al.*, 2003). Intra-uterine drainage is also a possibility (Funkquist *et al.*, 1983). The fertility may thus be preserved, although decreased, but with high probability of recurrence of the disease (Nelson, Feldman & Stabenfelt, 1982; Meyers-Wallen, Goldschmidt & Flickinger, 1986; Gilbert, Nöthling, & Oettle, 1989; Trasch, Wehrend & Bostedt, 2003; Gobello *et al.*, 2003).

The type of antimicrobial drug chosen for adjunctive pyometra treatment is mainly based on previous knowledge of antimicrobial susceptibility of *E. coli*. Studies performed in one country do not necessarily reflect the situation in another due to different traditions and regimes regarding the use of antimicrobials (Gandotra *et al.*, 1994; Yates, 1996; Pradhan *et al.*, 1999; Lee *et al.*, 2000; Wernicki, Krzyzanowski & Puchalski, 2002). In canines, most studies on *E. coli* are performed on isolates from urinary tract infections, another site from where *E. coli* also is the pathogen most commonly isolated (Low *et al.*, 1988). Bacterial cultures from urinary tract infections are likely to be biased towards problematic cases, and it is uncertain if their resistance pattern is representative also of *E. coli* from pyometra (Bywater, 2000; Kern *et al.*, 2002).

Endotoxin

Endotoxin (ET) is a lipopolysaccharide part of the outer cell wall of Gram-negative bacteria, such as *E. coli*. The ET is released into the circulation during vigorous bacterial growth or cell disintegration and interacts with inflammatory and endothelial cells (Devoe & Gilchrist, 1973). When present in the blood, ET induces a wide range of biological effects and symptoms such as fever, lethargy and elevation of heart and respiratory rates (Van Miert & Frens, 1968). Among the ET activities are complement and coagulation cascade activation, platelet activation, generation of vasoactive kinins, cytokines, oxygen free radicals, the arachidonic acid-derived prostaglandins, thromboxane and platelet activating factor (Morrison and Ryan, 1987). Moderate production of these mediators are beneficial for stimulation of the immune system and microbial killing (Rietschel & Brade, 1992). However, when released in large amounts ET can lead to fatal endotoxic shock with depression of the reticuloendothelial and circulatory systems (Goodwin & Schaer, 1989; Rietschel & Brade, 1992; Okano *et al.*, 1993). Endotoxins in the circulation can also directly generate endothelial damage, disseminated intravascular coagulation and generalised organ failure (McAnulty, 1983; Rietschel & Brade, 1992).

In normal physiological conditions, minor quantities of ET originating from the intestinal bacteria constantly transmigrate the bowel mucosa and enter the portal circulation. The ET is then filtered by the Kupffer cells and hepatocytes of the liver, preventing systemic endotoxemia (Nolan, 1981; Fox, Thomas & Broitman, 1990). Normally the ET clearance from the circulation occurs within minutes and symptoms only develop when the hepatic capacity is exceeded (Wardle, 1975).

Blood concentrations of ET have previously been related to outcome (survival or death) in cases of pyometra (Okano, Tagawa, & Takase, 1998). Bitches that died from the disease had significantly higher levels of ET (mean level 74.2 pg ml⁻¹) compared with the survivors (mean level 9.5 pg ml⁻¹). This suggests that presurgical measurement of ET levels could be used as a marker for severity of the disease and for determination of the chance of survival. In humans endotoxemia usually precedes septicemia (Van Deventer *et al.*, 1988). The early detection of endotoxemia, also in dogs, would allow for therapeutic intervention to prevent further development of septicemia. In another study, endotoxemia was detected prior to surgery in 15 bitches with pyometra, with tenfold higher mean levels than in the study by Okano and co-workers (1998), but all the bitches survived (Wessels & Wells, 1989). In contrast, endotoxemia was not consistently shown in two other studies of pyometra (Børresen & Naess, 1977; Fransson *et al.*, 1997). All these results suggest that ET is involved in the pathogenesis of pyometra, but not always detectable due to variations in blood concentrations and in procedures of sampling and analysis.

Prostaglandin F_{2α}

Prostaglandins are important in reproductive biology with many physiological and pharmacological roles and also as mediators in inflammatory events (Kindahl *et al.*, 1976; Kindahl, 1980; Fredriksson, Kindahl & Edqvist, 1985). Prostaglandins are derived from arachidonic acid and are produced and released from neutrophils, macrophages, lymphocytes and platelets during inflammation (Kindahl, 1980). The systemic release of prostaglandin F_{2α} (PGF_{2α}) can be followed by measurement of its more stable main circulating metabolite 15-keto-13,14-dihydro-PGF_{2α} (PG-metabolite) (Granström & Kindahl, 1982). The uterine endometrium is also able to synthesise and release prostaglandins and mainly PGF_{2α} (Heap & Poyser, 1975). Increased plasma levels of PG-metabolite have been measured in animals of several species, including the canine, with pathological inflammatory conditions of the uterus (Hughes *et al.*, 1979; Vandeplassche, Coryn & De Schepper, 1991; Kindahl *et al.*, 1992; Mateus *et al.*, 2003). A relation between plasma PG-metabolite levels and severity of uterine damage has been shown in cattle with post partum endometritis (Mateus *et al.*, 2003).

In addition, bacterial ET is a potent inducer of prostaglandin release from macrophages and mononuclear cells (Morrison & Ryan, 1987). The PG-metabolite has been shown to be a reliable and sensitive marker of experimentally induced endotoxemia in cattle, goats, pigs and horses (Fredriksson, 1984; Fredriksson,

Kindahl & Edqvist, 1985, Holst, Edqvist & Kindahl, 1993; Daels *et al.*, 1987). The PG-metabolite is, compared with ET, more stable, has a longer half-life and does not bind to sampling materials or proteins in plasma (Tobias, Soldau & Ulewitch, 1986). The role of prostaglandins in relation to endotoxin release in bitches with pyometra has not yet been determined.

Pyometra is one of the canine bacterial infections potentially at risk of progressing into the systemic inflammatory response syndrome (SIRS) (Hardie, 1995). Fulfilment of certain criteria, determined to predict SIRS, has previously been confirmed in over 50% of bitches suffering from the disease (Fransson, 2003). SIRS is the response to an initial local inflammatory reaction which has shifted through the cascade release of the endogenous inflammatory mediators to an uncontrollable systemic reaction where mediators directly stimulate other mediators and continue without the presence of the initiating agent (McAnalty, 1983; Purvis & Kirby, 1994). Various incidents such as severe trauma, infection or endotoxin can initiate a systemic inflammatory process which can be present with (septicemia) or without bacterial infection (Hardie, 1995). The presence of SIRS has been linked to lower survival rates and longer hospitalisation (Brady & Otto, 2001). Assessment of SIRS is therefore important to determine severity of the disease, optimise treatments and prevent fatal outcomes (Purvis & Kirby, 1994).

Aims of this study

The aims of the research presented in this thesis were to:

- Explore whether there are breed- and age-related differences in the incidence of pyometra in Sweden.
- Determine whether pyometra is caused by closely related *E. coli* clones and whether morphologically different colony types isolated from one uterus are genetically similar.
- Evaluate whether the infected uterus and the urinary tract in bitches with pyometra are infected with genetically related *E. coli* strains.
- Determine the antimicrobial resistance of *E. coli* strains isolated from pyometra and urinary tract infections and to explore whether antimicrobial resistance is a cause of recurrence of the pyometra after medical treatment.
- Evaluate whether data on antimicrobial resistance of *E. coli* from urinary tract infections can be used as a guide for drug selection in the treatment of pyometra.
- Explore whether endotoxemia is present in bitches with pyometra and study the possible use of hematological and blood biochemical parameters as markers for endotoxin release.
- Determine whether prostaglandin release is present in bitches with pyometra.
- Evaluate whether the PG-metabolite concentration can be used as a marker for the severity of the disease and whether such data is useful in the differentiation of pyometra and cystic endometrial hyperplasia.

Materials and methods

General descriptions of the materials and methods used in the studies presented in this thesis are described here. For further details, see papers I to V.

Animals

Bitches selected for the clinical studies were client-owned clinical cases at the Department of Small Animal Clinical Sciences, SLU, Uppsala, Sweden. The study periods were 1991-1994 and 1997 (II); 1991-1993 and 2002-2003 (III); 2001-2002 (IV, V). The studies were approved prior to the onset of any investigations by the Uppsala County Regional Ethical Board. The presumptive diagnosis pyometra was confirmed by clinical examination combined with radiology, or ultrasound or both, and by macroscopic and histopathological examinations (Wadås *et al.*, 1996; Fransson, 2003). In Study IV, bitches undergoing surgery for non-infectious reasons were used as a control group. Study V included clinically healthy, age-matched, intact bitches staying at the clinic as well as a group of bitches that had previously been enrolled in the study when ovariohysterectomised for pyometra.

Study designs

The purpose of Study I was to test the hypothesis that there are breed- and age-related differences in the incidence of pyometra in Sweden. To assess the incidence of pyometra in bitches, data obtained from the insurance company Agria Försäkringar AB (P.O. Box 70306, SE-10723 Stockholm, Sweden) were evaluated. In Sweden it is common practice to insure dogs and over 30% of all Swedish dogs are insured by this company (Egenvall *et al.*, 1999). The Agria data base included records on over 200,000 dogs for the years 1995 and 1996. There are two types of insurances that can be purchased separately or in combination: (1) veterinary care insurance, for which there is no age limit and (2) life-insurance, which is only valid up to ten years of age. Bitches up to ten years of age enrolled in both types of insurance in 1995 and 1996, respectively, were included in the study. The risks of pyometra were evaluated in thirty breeds represented by at least 800 bitches insured in both 1995 and 1996.

Study II was designed to evaluate the hypothesis that *E. coli* isolates from the uteri of bitches with pyometra are genetically similar. This would indicate the presence of particularly virulent bacterial clones and possibly also spread of these clones between animals. Eighty-four *E. coli* isolates from the uterus of 70 pyometra bitches were examined with a genotyping method. The genotypes of 96 different *E. coli* isolates within the uterus of ten other pyometra bitches were also studied. When morphologically different colony types could be isolated from the same bitch, all types were examined. In six bitches with pyometra and

simultaneous urinary tract infection, the evaluated *E. coli* strains were tested to determine whether they were genetically related, identical or different by means of the chosen method. The method with restriction enzyme analysis and pulsed-field gel electrophoresis (PFGE) is highly discriminatory and suitable for this type of epidemiological studies (Sternberg, 1998; Münnich & Lübke-Becker, 2004).

The objective of Study III was to test the hypothesis that data on antimicrobial susceptibility in *E. coli* bacteria from urinary tract infections are representative also for *E. coli* isolates from pyometra. Changes in the proportions of resistance in isolates from both urinary tract samples and pyometra, were also evaluated during a ten-year period. Antimicrobial resistance among *E. coli* isolates from the uteri of bitches with pyometra was determined and compared with data from diagnostic submissions of urine samples of dogs. The urine sample isolates were grouped according to origin (animal hospitals or smaller clinics). From the authors of a previous pyometra study (Franklin, Horn af Rantzen & Mörner, 1996), raw data (minimum inhibitory concentrations, MICs) were obtained and one isolate per bitch was randomly selected to enable comparison. Raw data (MICs) from all antimicrobial susceptibility tests of *E. coli* isolated from urine samples of dogs at the Department of Bacteriology, SVA, during the years 1991-1993 and 2002-2003 were retrieved from SVA.

Study IV explored the hypothesis that bitches with pyometra have increased plasma levels of endotoxin in the circulation; in addition it investigated possible haematological or biochemical markers for endotoxin release. Plasma endotoxin concentrations were measured in ten bitches suffering from pyometra and with moderately to severely depressed general attitude (more prone to suffer from the effects of endotoxemia). To increase the possibilities of detecting fluctuating endotoxin levels, blood samples were taken on five occasions before, during and after surgery. In addition, the prostaglandin $F_{2\alpha}$ metabolite 15-ketodihydro-PGF $_{2\alpha}$ (PG-metabolite) was analysed.

In Study V the hypotheses that PG-metabolite levels are elevated in bitches with pyometra and that they differ between bitches with pyometra and CEH were tested. PG-metabolite was evaluated in 58 bitches with pyometra, 11 bitches with CEH and 20 healthy controls. We had previously found that plasma levels of endotoxins were significantly increased in bitches with pyometra and that the endotoxin levels were correlated to the significantly elevated plasma PG-metabolite concentrations (Study IV). These results indicated that analysis of PG-metabolite could be a marker for endotoxemia and severity of the following systemic effects of the disease. To evaluate whether PG-metabolite analysis can act as a clinically useful marker for systemic inflammatory response syndrome (SIRS) in bitches with pyometra, a comparison of PG-metabolite levels in SIRS-positive and SIRS-negative cases was performed. The chosen criteria for SIRS-identification were the most sensitive alternatives applied for dogs (Hauptmann, Walshaw and Olivier, 1997). Possible correlations between PG-metabolite levels, clinical biochemical and hematological parameters, case history, clinical examinations and morbidity as measured by hospitalisation length, were also evaluated.

In addition to general notes on case history and results of the clinical examination the admitting clinician also determined the general attitude of the pyometra bitches to be moderately or severely affected, mucous membrane colour, dehydration status and whether the circulation was impaired (IV). In Study V the clinician filled out a form specifying rectal temperature, heart rate, respiratory rate, mucus membrane colour, capillary refill time, location for pain response at abdominal palpation, hydration status and general attitude on admission. After surgery the rectal temperature, heart rate and respiratory rate were recorded daily on a special form from which the length of hospitalisation was also determined (V). Information on treatment, complications to treatment and mortality (IV, V) were obtained from the medical records.

Sampling

Bacteriological examinations (II-IV)

Samples for bacteriological examination of the uterus were obtained immediately after ovariectomy as uterine biopsies (II, IV) or by inserting sterile fibre swabs (Culturette; Becton Dickinson AG) in the uterine lumen (III). For the uterine biopsies a 1x1 cm section of the uterine wall was aseptically removed, placed in a sterile vial and kept at 4 °C before culturing. In Study II the bacterial strains originated from uterine biopsies that were placed in thioglycolate medium prior to bacterial cultivation (Wadås *et al.*, 1996).

The urine samples used in Study II and IV were collected into a sterile syringe through cystocentesis during surgery. In Study II the urine sample was immediately transported to the former Department of Clinical Microbiology, SLU, for bacterial isolation and identification. In Study IV the urine was poured onto agar dip-slides (Uricult®, Orion Diagnostica, Espoo, Finland) designed to isolate the most common human urinary tract pathogens, and cultured for 16-48 h at 37 °C. If there was visible bacterial growth, the agar slides were transported to the Department of Bacteriology, SVA, Uppsala, Sweden, where isolation and identification was performed. In Study III, the collections of urinary tract *E. coli* strains originated from samples and dip-slide cultures submitted to the Department of Bacteriology, SVA, Uppsala, Sweden, from veterinary clinics and hospitals, for bacterial isolation and identification. These latter collections of urinary tract *E. coli* strains originated from urine of both male and female dogs (III).

Blood analyses (IV-V)

Blood samples for biochemical, hematological and PG-metabolite analysis were obtained by venopuncture of the distal cephalic vein immediately before surgery. EDTA, sodium-heparinised and non-additive vacutainer tubes (Becton-Dickinson, Stockholm, Sweden) were used for sample collection. Biochemical and hematological analyses were performed using routine methods, at the Department of Clinical Chemistry, SLU, Uppsala, Sweden. Sodium-heparinised plasma for PG-metabolite analysis (Study IV and V) and hormone (progesterone and

oestradiol-17 β) analysis (Study IV) was stored at -20°C until assayed at the Department of Obstetrics and Gynaecology and the Department of Clinical Chemistry, SLU, Uppsala, Sweden, respectively.

Samples for plasma endotoxin analysis were obtained on five different occasions: (1) before induction of the general anaesthesia, (2) during skin incision, (3) immediately after uterus removal, (4) during skin suturing and (5) the day after surgery. Endotoxin-free vacutainer tubes containing sodium heparin (EndoTube ET®, Haemochrome AB, Göteborg, Sweden) were used to collect the blood samples. After sampling the tubes were immediately placed on ice and next centrifuged. The serum was then transferred to endotoxin-free glass tubes and stored at -20°C, until transportation.

Laboratory analyses

Bacterial isolation, identification of E. coli and serotyping (II-V)

The bacterial isolations and identifications in Study II, III and IV were performed according to similar standard techniques at the former Department of Clinical Microbiology, SLU, Uppsala, and the Department of Bacteriology, SVA, Uppsala, Sweden (Holt *et al.*, 1994; Barrow & Feltham, 1993). The uterine biopsies (II, IV) and fibre swab samples (III) were cultured on MacConkey agar plates (Oxoid, Basingstoke, UK) with subculture of morphologically typical *E. coli* isolates on horse blood agar (5% v/v) and bromchresol-purple lactose agar. All isolates of *E. coli* were identified by positive reactions for indole and p-nitrophenyl- β -D-glucopyranodiduronic acid. Twenty-five strains of *E. coli* from 17 bitches with pyometra (collection A), in Study II, were serotyped by the Division of Diagnostics, Statens Serum Institut, Denmark. The production of α -hemolysin and presence of mannose-resistant or sensitive hemagglutination was also determined.

Pulsed-field gel electrophoresis (II)

The DNA plugs were moulded according to the method described by Christensen and co-workers (1994) with some modifications. The freeze-stored *E. coli* cultures were thawed and subcultured twice before inoculating a single colony into 2 ml of Luria-Bertani (LB) broth. After 24 h incubation at 37 °C in an incubator shaker, growth was profound in all broth cultures. Viable count was performed on selected bacterial strains. The gel plugs were incubated at 56 °C for 20 h with proteinase K (Boehringer Mannheim, 1 mg/ml buffer). The restriction enzyme Xba 1 (MBI Fermentas, Hannover, USA) was used for the DNA digestion. The Lambda DNA-PFGE Marker (Pharmacia Biotech, Piscataway, USA) was added as a molecular size marker on double lanes of each gel as was also a typed ATCC-strain of *E. coli* (ATCC No. 25922), in a minimum of two double gel lanes, to enable comparison among the different gels. The electrophoresis chamber (Gene Navigator System, Pharmacia, Biotech, Uppsala, Sweden) was used and the gel was run in a clamped homogenous electrical field (CHEF) system in six phases for a total of 22 h (5 s pulses for 3 h; 9 s pulses for 5 h; 12 s pulses for 5 h; 20 s pulses

for 4 h; 25 s pulses for 3 h; 30 s pulses for 2 h). After electrophoresis, the gel was stained in ethidium bromide solution (final concentration 0.4 µg ml⁻¹) for 30 min and photographed under 312 nm ultraviolet light. All strains were run in at least two lanes (double lanes) on each gel to avoid errors due to variability of the method. All DNA profiles on the gel photographs were examined by visual comparison and scanned by a flatbed scanner.

Antimicrobial susceptibility tests (III)

Susceptibility to antimicrobials was investigated using a commercially available microdilution system (VETMIC®, SVA). For the uterine isolates, microdilution panels with antimicrobials intended for monitoring of resistance were used (Ampicillin, Apramycin, Ceftiofur, Chloramphenicol, Enrofloxacin, Florfenicol, Gentamicin, Nalidic acid, Neomycin, Streptomycin, Sulphamethoxazole, Tetracycline, Trimethoprim). For the isolates from urine samples, break-point panels with a more limited range of antimicrobials, intended for clinical diagnostics, were used (Ampicillin, Chloramphenicol, Enrofloxacin, Gentamicin, Nitrofurantoin, Streptomycin, Sulphamethoxazole, Tetracycline, Trimethoprim, Trimethoprim-sulphamethoxazole). The determination of MICs was performed according to the standards of the National Committee of Clinical Laboratory Standards (NCCLS, 2000; NCCLS, 2002) using cation-adjusted Mueller-Hinton broth (Oxoid) with *E. coli* ATCC 25922 as a quality control. For all collections of isolates, microbiological criteria were used to define resistance (EUCAST, 2000; Kahlmeter *et al.*, 2003). An isolate was regarded as resistant to a specific antimicrobial when the MIC was higher than the highest MIC of the wild-type population (inherently susceptible population).

Endotoxin, PG-metabolite and hormone analyses (IV-V)

All freeze-stored samples for endotoxin determination were transported, cooled with freeze clamps, and arrived within four hours at the commercial laboratory Scan Dia Laboratory Services, Charlottenlund, Denmark, where the analyses were performed. The samples were analysed with a kinetic turbidimetric Limulus amoebocyte lysate (LAL) assay approved for endotoxin determinations by the Food and Drug Administration, USA (1987). At the Department of Clinical Sciences, SLU, Uppsala, Sweden, a radioimmunoassay (RIA) was used to analyse 15-Ketodihydro-PGF_{2α} (PG-metabolite) in duplicate (Granström & Kindahl, 1982). Progesterone was analysed using an enhanced luminescence immunoassay (Immulite, Diagnostic Products Corporation, Los Angeles, CA, USA). Plasma oestradiol (E2-17β) concentration was determined using a modified double antibody RIA kit (Diagnostic Products Corporation, Los Angeles, CA, USA).

Data analyses

In Study I, dogs were considered pyometra cases if the owner had reimbursed claims for pyometra for veterinary care, and/or life irrespectively if they were reimbursed or not. Analyses for 1995 and 1996 were carried out separately. The crude and breed-specific median ages were calculated and the crude and breed-

specific median ages at developing pyometra were determined. Twelve-month crude and age-specific risks (cumulative incidences, incidence proportions) were determined. For the 30 breeds with at least 800 bitches insured at the beginning of the years 1995 and 1996, breed-specific and breed-and age-specific 12-month risks were determined for each year. All the 12-month risks presented were adjusted.

Because few Swedish bitches are neutered (Egenvall *et al.*, 1999) and because neuter status was not included in the database, it was decided to adjust the number of dogs at risk in each age group, using the 12-month risk of pyometra in the previous age categories, as follows: The denominator in the first age category was un-adjusted. The denominator in the second age category was adjusted by the 12-month risk of pyometra in the first age category (un-adjusted denominator_{2nd age category})*(1-risk_{1st age category}) and the denominator in the third was adjusted by the 12-month risk of pyometra in the first and second category (un-adjusted denominator_{3rd age category})*(1-risk_{1st age category})*(1-adjusted risk_{2nd age category}), and so on. Age- and breed-specific adjusted 12-month risks were used to estimate the cumulative crude and breed-specific risk of pyometra in each age category, using a combined risk formula: total risk in *n*th age category=1-(1-risk age category <1)*(1-risk age category 1<2)*...*(1-risk age category *n*) (Kleinbaum, Kupper & Morgenstern, 1982).

Multivariable analysis was conducted to simultaneously evaluate the effects of breed, location, urban versus rural environment and age upon the incidence of pyometra. In logistic regression analyses, breeds were represented by 29 dummy variables with mixed-breed dogs as the initial baseline.

Since the use of chronological age to adjust for disease risk has its limitations (i.e. a 7-year-old poodle is not as physically “old” as a 7-year-old Bernese Mountain Dog), biological age for a given breed was defined as 0 to <10% mortality (age I), 10 to <20% mortality (age II), 20 to <30% mortality (age III), 30 to <40% mortality (age IV), and ≥40% mortality (age V), using data for all dogs from 1996 (male and female) (Egenvall, *et al.*, 1998). Death estimates were calculated on all deaths regardless of whether they were caused by disease, trauma or euthanasia for non-medical reasons.

In Study I, four sets of multivariable models were developed. In model I, all main effects except age were evaluated. Model II was the same as model I with all possible 2-way interactions evaluated. Model III was the same as model II, with the age variable included, but interactions with age were not tested. In the 4th model, interactions with age were tested. Models were reduced based on the change in deviance. The statistical software program SAS (SAS Institute, Cary, NC) was used to analyse the data, and the procedure GENMOD was used for logistic regression.

In Study II, gel normalisation and cluster analysis was performed with the software GelCompar (Applied Maths, Kortrijk, Belgium). In all gels, one double lane of the ATCC strain (ATCC No. 25922) was used as a standard for the normalisation of the gel. The other double lane of the ATCC strain was used as a

control for normalisation of the gel and for calculations of the reproducibility of the method.

In Study III-V the software Statistica (Version 6.0, StatSoft Inc., Tulsa, USA) was used for performance of the analyses. The Pearson Chi-square statistic was used to assess observed differences in proportions of antimicrobial resistance between different subsets of isolates.

In Study IV, a repeated measures ANOVA was used to test for differences in endotoxin means by patient group (control and pyometra) with sampling occasion of endotoxin level in plasma as repeated measures variable. A second repeated measures ANOVA was performed to test for differences in endotoxin means by general condition and sampling occasion within the pyometra patient group. Unpaired t-tests were used for groupwise comparisons of means of the two patient groups and endotoxin means of antibiotic-treated and untreated bitches. Pearson's product moment correlation coefficient (r_p) was calculated between endotoxin concentrations, PG-metabolite concentrations, other blood chemistry parameters and body temperature.

A one-way ANOVA with a post-hoc Tukey test was used to test for differences in PG-metabolite levels by patient group (control, pyometra and CEH) in Study V. Fisher's exact test, Spearman's rank correlation coefficient (r_s) and Pearson's product moment correlation coefficient (r_p) were used to test for associations between nominal- or ordinal-scale variables and interval-scale variables, respectively. Binary logistic regression analyses were used to evaluate blood parameters in relation to controls and bitches with pyometra or CEH. The regression models were tested for sensitivity and specificity. T-tests were used to test for differences in PG-metabolite levels between SIRS-positive and SIRS-negative cases. This was tested for (1) a group consisting of both pyometras and CEHs, (2) the pyometra group, and (3) the CEH group.

For further details, see paper I-V, respectively, in the appendices.

Results and discussion

The incidence of pyometra is breed- and age-dependent

Breed dependency

The results of the present thesis verify that pyometra is an important disease in intact bitches. Overall, almost 25% of the insured dog population had developed pyometra by 10 years of age. In the three breeds at highest risk of developing the disease in 1996 (rough-haired Collie, Rottweiler, Bernese Mountain Dog), approximately 50% of the bitches had experienced pyometra before reaching 10 years of age (Table 1). The percentage of bitches that developed pyometra (judged by recorded reimbursement) before 10 years of age, in all 30 breeds included in the present study is shown in Table 1.

Altogether, 1803 and 1754 bitches in 1995 and 1996 respectively, had claims for pyometra as recorded by reimbursed veterinary care claims, claims settled for life insurance, or both. The case fatality for pyometra was 4.3% in 1995 and 4.2% in 1996, including both deaths and elective euthanasia. If it would have been possible to monitor the risk of pyometra also in older bitches, i.e. over 10 years of age, these figures would most likely be slightly higher. Not that age itself leads to an increased risk for fatal surgical complications in a healthy animal (Wheaton *et al.*, 1987), but the risk for other concurrent diseases increases with age thus increasing the risk of surgical complications or the owner's choice of euthanasia.

The crude 12-month risk of pyometra (the risk of a bitch developing pyometra during a specific year) for bitches less than 10 years of age, was 2%. The crude median age at development of pyometra in 1995 and 1996 was 6.5 and 6.9 years, respectively, but the true median age for pyometra in the general population including bitches of 10 years and older is probably higher (Niskanen & Thrusfield, 1998). In Study III of the present thesis, the mean age for the sampled pyometra bitches was 8.6 years and in Study V it was 9.9 years, which is similar to previous studies (Dow, 1958; Hardy & Osborne, 1974). Interventions with for instance steroid hormone compounds may increase the risk of pyometra even at younger ages (Bowen *et al.*, 1985; Niskanen & Thrusfield, 1998). In Sweden it is uncommon to administer steroid hormones such as oestrogen substances (the only drug registered for use against mismating in Sweden during 1995 and 1996) because of the risk of developing pyometra.

Breeds with high risk of pyometra

Breeds with high risk of the development of pyometra in the present study were Collie (rough-haired), Rottweiler, Cavalier King Charles Spaniel, Bernese Mountain Dog and Golden Retriever. The risks for the 30 breeds in the present study compared with risks for these breeds published in the studies by Krook, Larsson & Rooney (1960), Ewald (1961) and Niskanen & Thrusfield (1998) are shown in Table 1.

Table 1. Selected data of the 30 dog breeds investigated in the present study; showing total percentage of bitches up to 10 years of age reimbursed for pyometra, the risk of pyometra in the present study compared to the baseline, adjusted 12-month risk of developing pyometra in 1996 and references to the predisposition (A, B) and risk (C) in previous studies. Normal risk ↔; increased risk ↑; decreased risk ↓; NA= not analysed separately. A = Krook, Larsson & Rooney (1960); B = Ewald (1961); C = Niskanen & Thrusfield (1998).

Breed	Pyometra (%) before 10 years of age	Risk in the present study	Risk (%) in year 1996	Risk or predisposition according to references
Bernese Mountain Dog	54	↑	3.9	C: ↔
Rottweiler	50	↑	4.4	AC: ↑
Collie, rough-haired	46	↑	4.5	ABC: ↑
Cavalier King Charles Spaniel	41	↑	3.8	C: ↑
Golden Retriever	35	↑	3.3	C: ↑
Shetland Sheepdog	30	↔	2.4	
German Pointer	29	↔	0.9	
Boxer	28	↔	2.7	A: ↓
Jämthund	28	↔	2.4	
English Cocker Spaniel	27	↑	2.8	
West Highland White Terrier	26	↔	2.5	C: ↔
English Springer Spaniel	25	↔	1.9	C: ↔
German Shepherd Dog	25	↓	1.9	
Mixed breed	23	NA	NA	C: ↓
Labrador Retriever	22	↔	1.8	C: ↔
Gråhund	17	↔	1.3	
Flat Coated Retriever	17	↔	1.4	
Drever	17	↓	1.6	
Swedish Hound	16	↓	1.2	A: ↑
Papillion	16	↔	1.5	
Cairn Terrier	15	↔	1.6	
Finnish Hound	15	↔	1.4	
Yorkshire Terrier	15	↔	1.3	
Poodle, miniature/toy	14	↔	1.6	AB: ↓
Dachshund, miniature	13	↔	0.3	
Dachshund (normal size and wirehaired)	12	↓	1.1	AB ↓, C: wirehaired ↓
Border Collie	12	↔	0.7	
Bichon Frisé	11	↔	1.2	
Bearded Collie	10	↔	0.9	
Beagle	10	↔	0.7	

Breeds with low risk of pyometra

Breeds which had lower risk of developing pyometra in the present study were the Swedish Hound (models III and IV, 1995), normal size Dachshund (all models, 1995) and miniature Dachshund (all models) (Table 1). Lower risk was also found for the German Shepherd Dog and the Drever when adjusted for biological age (models III and IV, both years). A reduced risk for mixed-breed dogs has been reported (Niskanen & Thrusfield), but mixed-breed dogs were used as a baseline for multivariable analysis in the present study and thus only separately analysed in the stratified analysis.

Two of the cited studies are 20-40 years old, and one study was performed in the last decade (Ewald, 1961; Krook, Larsson & Rooney, 1960; Niskanen & Thrusfield, 1998). Ewald (1961) and Krook, Larsson & Rooney (1960) compared hospital cases and Swedish cases of pyometra from post mortem examination cases, respectively. These authors compared dogs with and without pyometra in their analyses. The external validity in studies based on post mortem examination cases might be low because such studies are prone to selection bias. Niskanen & Thrusfield (1998) used affected dogs and controls from primary care practices, a method that achieves high external validity. The base population was not considered in any of these studies. In the present study there was complete information on the base population but less complete information on the cases than in the other studies (i.e. it was only stated that the bitches were diagnosed with pyometra).

Age dependency

When biological age was added, most breed-specific risks decreased with the exception of the Golden Retriever, which increased. This breed clearly has an increased risk of developing pyometra and the effect is even more evident when adjusted for biological age since few Golden Retrievers die early. Two breeds entered the multivariable models when adjusting for biological age, the German Shepherd and the Drever, which then were implicated with lower risk of developing pyometra compared with the baseline. The low risk might reflect the fact that many dogs of these breeds die early (e.g. Drevers which are hunting dogs often die because of trauma). The results might have differed and these breeds been included in the baseline if the biological age variable had included only deaths due to disease (as opposed to deaths from trauma or disease).

The interactions with biological age could be interpreted as that in some breeds (e.g. Rottweiler, rough-haired Collie, Golden Retriever, and Cavalier King Charles Spaniel in 1995), the risk of pyometra actually increases more and at an earlier age compared with other breeds. These breeds may carry a higher genetic predisposition for pyometra than other breeds. In general, breed differences may reflect true genetic differences or merely constitute a reflection of the different life spans in different breeds. If true genetic differences in predilection exist, the possibility of instituting breeding programmes to control the disease could be considered. Bernese Mountain Dogs were only at an increased risk in the models I and II, but dropped out when adjustments for biological age were made. The

reason that Bernese Mountain Dogs developed pyometra to the extent they did was probably because of the relatively high mortality before 10 years of age in that breed and not a true increased risk of pyometra in general. Some owners may want to neuter their bitches at an early age to prevent the development of pyometra. Given the different age patterns for risk, the maximum or optimal age for elective spaying differs among breeds. It is likely that the predisposition for pyometra is similar in other countries but that the common practice of neutering at an early age prevents recognition of the true disease frequency.

Substantial genotypic variation in pyometra-inducing *E. coli*

Bacterial epidemiology (II)

The chromosomal restriction polymorphism which could be resolved by pulsed-field gel electrophoresis (PFGE), was sufficient for a consistent differentiation of the various isolates, and strains from the 80 different bitches showed different DNA restriction fragment profiles (Fig. 2). The repeated examinations of the control strain showed a high reproducibility of the method between different gels. The variations of DNA-profiles of *E. coli* isolates from the uterus of different bitches with pyometra indicate that pyometra is caused by *E. coli* originating from the normal flora of the bitch and not by certain clones spread between animals.

In the bitches where examination of several (8-16) colonies of *E. coli* from the uterus was performed, all isolates from the same bitch showed identical DNA profiles. This indicates that in cases of *E. coli* pyometra, only one bacterial clone establishes itself in the uterus, multiplies and acts as the main agent in the disease. One genotype is therefore predominant in the uterus. This likely bacterial pathogenesis is in accordance with a study of human urinary tract infections by Arbeit and co-workers (1990) who showed by genotyping that the *E. coli* infection within a given patient is due to a single bacterial clone.

The morphologically different mucoid and non-mucoid colonies found in some bitches were of the same genotype with identical DNA profiles in 16 of the 17 bitches assayed. However, one strain from a pyometra bitch had one band located in different sites in the two forms of the strain. Since the PFGE was repeated and performed with double samples, it is not likely to be a difference caused by a methodical alteration.

Isolates with identical PFGE patterns are considered to be clonal (Maslow, Slutsly & Arbeit, 1993; Tenover *et al.*, 1995). Isolates with shifts of two or three bands are considered to be closely related although a point mutation, an insertion, a deletion or a chromosomal inversion has occurred. One single genetic event can give rise to up to three band differences, and therefore four or more bands should differ before an isolate is assigned to a different clone (Tenover *et al.*, 1995). Others consider isolates as different strains when three or more bands are dissimilar (Maslow Slutsly & Arbeit, 1993).

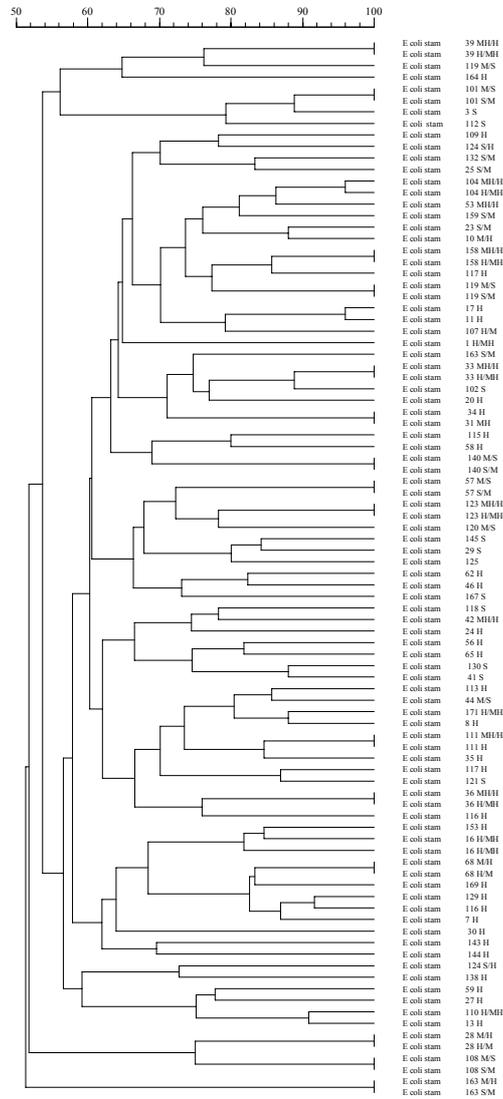


Fig. 2. Dendrogram showing relations between DNA-profiles of *E. coli* isolates from pyometra cases. If more than one colony type was isolated from one bitch, the identification number of that bitch and the colony type characteristics (M = mucoid, S = smooth, H = smooth and hemolytic, MH = mucoid and hemolytic) for both isolates are listed. Isolates no. 31 and 34 are from the same uterus.

In our study, however, the different profiles of the DNA fragments between the mucoid and non-mucoid forms in one bitch never altered their type in more than one band, and can thus be considered to be the same clone. Contrary to expectations, in all but the case mentioned above, no evidence of the morphological differences was visible in the DNA-profile of the two colony types. This could be explained by the fact that a mutation has occurred, but lies within an area of the genome which is unaffected by the restriction enzyme Xba 1, and therefore was undetectable in this study. In this case the dissimilarity might appear if the procedure was repeated with other restriction enzymes. However, it is more likely that the genomes of the two forms are in fact identical and the only difference is whether their capability to produce hemolysin or mucopolysaccharide is being expressed or not. Different morphological types of the same bacterial strain, mimicking mixed growth, have been shown in *E. coli* from urinary tract infections in man (Nichols, 1975). An advantage with PFGE is that genetic events which do not alter the main strain phenotype can be visible in the DNA fragment pattern if they change the mobility of a DNA-fragment. Point mutations occurring on an enzymatic cleavage site would also be detectable, however unlikely to occur in this case.

Another observation was that *E. coli* isolates from the uterus and the urinary tract of the same animal were indistinguishable in the bitches with pyometra and simultaneous urinary tract infection (Study II). These results indicate that in cases of urinary tract infection and *E. coli* pyometra, the urinary tract and uterus are infected with the same bacterial strain (Sandholm, Vasenius & Kivistö, 1975; Järvinen, 1981; Wadås *et al.*, 1996). Given that *E. coli* strains isolated from both pyometra and urinary tract infections have many potential virulence features in common (Sandholm, Vasenius & Kivistö, 1975; Senior, DeMan & Svanborg, 1993; Wadås *et al.*, 1996; Yuri *et al.*, 1998; Chen *et al.*, 2003), the infected urinary tract could supply pathogenic bacteria, invading the uterus when conditions are favourable.

Prevalence and virulence attributes (II-IV)

E. coli were isolated from 66% of the samples from pyometra bitches during the study period 2002-2003 in Study III. In Study IV the corresponding figure was 90%. This dominance of *E. coli* is in accordance with previous studies on pyometra (Fransson *et al.*, 1997; Dhaliwal, Wray & Noakes, 1998). Abundant growth of *E. coli* was isolated from 20% of the urine samples of the pyometra bitches in Study IV, but all other urine samples showed no bacterial growth. In Study II, collection A, 74% of the *E. coli* strains had smooth colony morphology whereas 26% were mucoid. On horse blood agar, 61% of the *E. coli* strains were hemolytic. Among the 96 isolates from ten bitches in collection C, mucoid, smooth, hemolytic and non-hemolytic colonies were present on the primary culture plates and chosen for analysis.

Twenty-five strains of *E. coli* from 17 bitches with pyometra (collection A), in Study II, were serotyped in addition to genotyping (R. Hagman, unpublished data). The O-serotypes most commonly encountered were O4 (5 bitches), O6 (4 bitches)

and O2 (3 bitches). O22, O76/O83, O18, O76, and O-autoagglutination were found in one bitch each. In the five bitches from which two morphologically different strains were isolated, both were of the same O-serotype. The finding that pyometra *E. coli* commonly are clustered in serotypes O6, O4 and O2 is in accordance with the results of other studies (Grindlay, Renton & Ramsay, 1973; Sandholm, Vasenius & Kivistö, 1975; Dhaliwal, Wray & Noakes, 1998; Bigliardi *et al.*, 2004). Capsule (K) antigens were found in 68% of the total number of strains, and in all bitches except 2 (88%). The presence of K-antigen is a well-known virulence factor in urinary tract infections in man (Kalmansson *et al.*, 1975). However, in two of the five bitches from which two strains were isolated, capsular antigen was not present on both strains. Flagellar antigen was found in 68% of the strains. Another common trait was the mannose resistant hemagglutination (MRHA), associated with the presence of P- and S-fimbriae, which was found in strains from 70% of the bitches. Type-1 fimbriae and the FimH adhesin are important for epithelial attachment and colonisation of uropathogenic *E. coli* in man, and as targets for protective vaccines (Langermann *et al.*, 2000). Future studies determining *E. coli* virulence factors with the goal to explore possible prevention of *E. coli* attachment and infection by vaccination also in canine UTIs and pyometra, is an interesting progression. The results from the present serotyping indicate that the *E. coli* strains from the uterus of one bitch are of the same genotype but can be of different phenotypes and even serotypes depending on their gene expression. In serotype studies of pyometra, the selection of bacterial colonies from the initial culture plate does not affect the O-serotype grouping but certainly does influence the presence of other bacterial antigens.

Antimicrobial resistance is unlikely to cause therapy failure

Pyometra isolates

The proportions of resistance to different antimicrobials in pyometra *E. coli* were low and of similar magnitude in both study periods (Table 2). Resistance to ampicillin was the most commonly observed trait among uterine isolates from 2002-2003, and also in urine isolates from hospitals from both study periods. This is consistent with beta-lactam antibiotics, mostly aminopenicillins, being the most commonly used class of antimicrobials in dogs (Odensvik, Grave & Greko, 2001). When comparing pyometra isolates with isolates from urine samples submitted from animal hospitals in 2002-2003, significantly lower proportions of resistance to ampicillin, streptomycin and tetracycline were recorded (Fig. 3). However, no differences were observed when comparing pyometra isolates and urine isolates from animal clinics. General data on antimicrobial resistance in bacteria from urinary tract infections cannot be used as a guide for selection of antimicrobials for treatment of pyometra.

In medical treatment of pyometra, antibacterial therapy is adjunctive to treatment with drugs such as prostaglandins or antiprogesterins. In pyometra studies the duration of treatment varies from a few days (Gilbert, Nöthling & Oettle, 1989) to four weeks or more (Meyers-Wallen, Goldschmidt & Flickinger, 1986).

Reported recurrence rates are generally high (Meyers-Wallen, Goldschmidt & Flickinger, 1986; Nelson, Feldman & Stabenfeldt, 1982; Gilbert, Nöthling & Oettle, 1989; Gobello *et al.*, 2003). Meyers-Wallen, Goldschmidt & Flickinger (1986) suggested that recurrence may be a clinical manifestation of the same infection, rather than re-infection with a new bacterial strain. Based on the results of the present study, resistance to antimicrobials is not likely to be a major explanation for therapy failure. Other aspects, such as the pharmacodynamics of the chosen drugs, are more likely to influence the outcome in terms of antibacterial effect. Rekha & Krishnappa (2000) suggested that the bacterial growth inside the uterus is mainly in the form of biofilms. In such infections, both the slower growth rate of the infectious agent, and the diffusion barrier of the biofilm matrix itself must be considered when choosing the drug, the dosage and the duration of treatment (Gristina *et al.*, 1987). In this study, susceptibility in isolates of *E. coli* from pyometra was mostly high, but multiresistance was also recorded, and therefore it is advisable to base selection of antimicrobials on culture and susceptibility tests. To further optimise the medical treatment of pyometra, more information on pharmacokinetic and pharmacodynamic relationships of antimicrobials in the diseased uterus is needed as a basis for selection of drug and dosage regimens.

Table 2. Cut-off values for resistance for 10 different antimicrobials and percentage resistance to these antimicrobials among *Escherichia coli* from pyometra and urine samples for two study periods. Pyo1 = pyometra samples 1991-1993, Pyo2 = pyometra samples 2002-2003, Uri1 = urine samples 1991-1993, Uri2 = urine samples 2002-2003. - = not tested.

Antimicrobial	Cut-off Values ^a (mg l ⁻¹)	Pyo1 n = 56 (%)	Uri1 n = 96 (%)	Pyo2 n = 80 (%)	Uri2 n = 205 (%)
Ampicillin	>8	7	22	10	15
Chloramphenicol	>16	2	7	0	-
Enrofloxacin	>0.25	2	11	4	8
Gentamicin	>8	0	3	0	1
Nitrofurantoin	>32	2	2	-	2
Streptomycin	>32	9	21	5 ^b	13
Sulphametoxalate	>256	-	-	8	-
Tetracycline	>8	9	23	4 ^b	10
Trimethoprim	>8	-	-	2	-
Trimethoprim-sulphamethoxazole	>1	4	10	-	14 ^c

^aValue above which the isolate was considered resistant, ^b79 isolates tested, ^c204 isolates tested.

Urinary tract isolates

In 1991-1993, 96 isolates of *E. coli* had been tested for susceptibility at SVA. The corresponding figure for 2002-2003 was 205. Among *E. coli* from all urinary samples from 1991-1993, the proportions of resistance to ampicillin, streptomycin and tetracycline were above 20% (Table 2). The corresponding figures for 2002-2003 were more than five percentage units lower, but when the data set was stratified by origin of the *E. coli* strains from animal hospitals or animal clinics there was no difference over the ten-year-period within the groups.

In the present study, it is probable that an increased number of animals investigated at animal hospitals suffered from complicated urinary tract infections as compared with those attending animal clinics. The former group is more likely to have been exposed to previous antimicrobial treatment that may have selected for resistant bacterial strains, which could explain the higher proportions of resistance to ampicillin and streptomycin. This has been shown in humans where significantly higher resistance proportions are found among *E. coli* isolated from complicated urinary tract infections, as opposed to uncomplicated cases (Kerrn *et al.*, 2002).

Other antimicrobials commonly used to treat urinary tract infections are trimethoprim-sulphonamides and fluoroquinolones. The proportion of resistance to fluoroquinolones among isolates from urine samples was 7-12% (Table 2). The cut-off values chosen for this study are low compared to break-points recommended by, e.g., NCCLS (2002). Nonetheless, isolates with MIC >0.25 mg l⁻¹ have a decreased susceptibility compared with inherently susceptible strains, most probably because of at least one mutation in one of the genes encoding the target enzymes of this class of drugs (Webber & Piddock, 2001). Treatment of infections with such isolates with fluoroquinolones may well be successful, but there is a risk for further mutations to higher levels of resistance (Drlica, 2003).

Multiresistance

Four pyometra isolates from each study period were resistant to more than one antimicrobial. Seven of these isolates were resistant to both ampicillin and streptomycin, and six of these also to other drugs. In the urine samples, resistance to three antimicrobials or more (multiresistance) was observed in 15% and 9% of the isolates from 1991-1993 and 2002-2003, respectively. The most common combination was resistance to ampicillin and streptomycin, observed in 11% of the 301 isolates and of these, 78% were multiresistant.

Multiresistance, with resistance to tetracycline, streptomycin and ampicillin being a common combination, has been reported in studies on resistance in *E. coli* isolated from faeces of healthy dogs and is mostly transferred by conjugation (Monaghan, Tierney & Colleran, 1981; Moss & Frost, 1984). The normal flora of the animals may thereby act as a reservoir from which resistance can be transferred to other, more virulent, bacteria of dogs. The risk of spread of resistance genes to bacteria colonising other animal species or people is difficult to

assess, but needs to be considered (Sternberg, 1999; Münnich & Lübke-Becker, 2004).

Pyometra is associated with endotoxemia ...

Bitches suffering from pyometra had significantly higher plasma levels of endotoxins than the control bitches (Fig. 3). The mean endotoxin levels (of all sampling occasions) were 28 pg ml⁻¹ (range 14 to 52 pg ml⁻¹) in the control group and 49 pg ml⁻¹ (range 20 to 123 pg ml⁻¹) in the pyometra group. Thus, the ET concentrations in the present study are in the same order of magnitude as in the study by Okano, Tagawa & Takase (1998), but ten-fold lower than those reported by Wessels & Wells (1989). In samples taken immediately after removal of the uterus or at the end of the surgical procedure a significant difference in mean endotoxin levels was shown between the two patient groups. However, if sampling were to have taken place on only one occasion, no significant difference in mean endotoxin levels between the two patient groups would have been detected for the samplings before surgery, during skin incision or the day after surgery. The measurable differences in mean values for these sampling occasions were not statistically significant because of a large variation in the data.

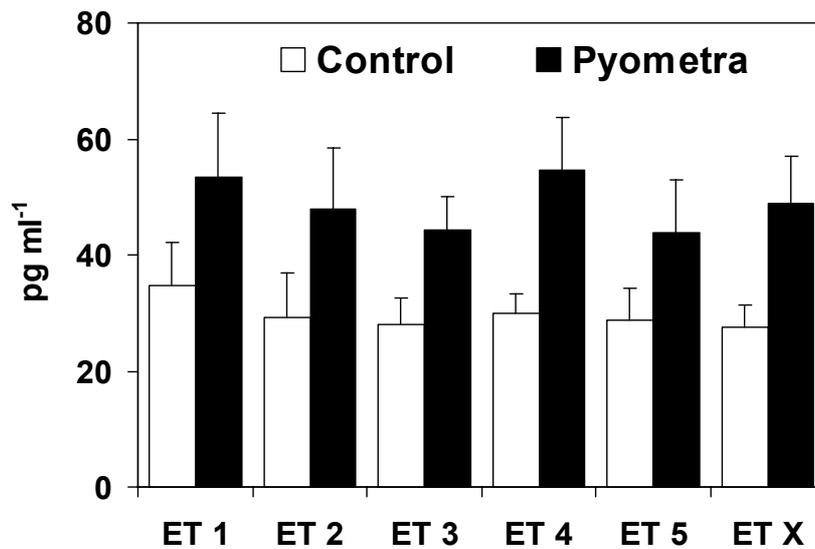


Fig. 3. Plasma endotoxin levels in the control group and the pyometra bitches measured at five different sampling occasions; ET 1 = before general anaesthesia, ET 2 = during skin incision, ET 3 = after removal of the uterus/major part of the surgical procedure, ET 4 = during skin suturing, ET 5 = the day after surgery, ET X = mean endotoxin levels of all sampling occasions. Error bars represent 1 SE of the mean. The differences in endotoxin levels were significantly ($P < 0.05$) higher in the pyometra bitches for sampling occasion ET 3, ET 4 and also for ET X.

The variations in ET levels between different studies of pyometra probably depend on study design, i.e. sampling procedures and case selection. Correct sampling technique and handling of the plasma samples is important to avoid contamination or binding of the ET to plasma proteins or sampling materials (Warren *et al.*, 1985; Tobias, Soldau & Ulevitch, 1986). Differing results between studies (and sample occasions) may also depend on timing of the sampling considering the rapid clearance of ET from the circulation (Van Deventer *et al.*, 1987; Fox, Thomas & Broitman, 1990). Frequent sampling is therefore an advantage in the evaluation of endotoxemia compared with analysis at only one point in time.

The biological effects of endotoxins *in vivo* not only depend on the amount of ET present in the blood but also on the susceptibility of the host (Galanos *et al.*, 1988). The sensitivity to ET varies between different animal species (McCuskey *et al.*, 1984) and also between individuals within a species (Van Miert & Frens, 1968). Higher ET susceptibility could explain why ET levels were not different in the bitches with severely depressed general condition when compared with those that were only moderately depressed. The rate of ET release also influences its effects. A certain tolerance to ET can be induced, where the individual response becomes less pronounced after repeated injections of the same amount of ET (Holst, Edqvist & Kindahl, 1993). As for prognostic value of endotoxin levels, all of the studied bitches survived in spite of six pyometra bitches having endotoxin concentrations $>75 \text{ pg ml}^{-1}$ on at least one sampling occasion implicating a poor prognosis (death) according to the study by Okano, Tagawa & Takase (1998).

In Study IV significant correlations were found between endotoxin concentrations (means of the five sampling occasions) and PG-metabolite, hemoglobin (Hb), packed red blood cell volume (PCV), lymphocytes, albumine and γ -globuline concentrations. The parameter that was correlated to ET levels as well as to most other features was PG-metabolite. This is not surprising as the potency of prostaglandins in inflammation and endotoxemia is well known (Bottoms, Johnson & Roesel, 1983). Since PG-metabolite is an accurate measurement of ET-induced prostaglandin synthesis and release in other species, the detection of PG-metabolite could be helpful in detecting endotoxemia also in dogs. The metabolite is more stable in plasma than ET, no prostaglandin-binding substances have been shown to be present in blood and the assay is more cost-efficient than the present method for ET analysis. Unfortunately, a method is not yet available for routine use in veterinary clinics. The parameters Hb, PCV and γ -globulins are not as specific for ET involvement as PG-metabolite since they may also be affected by dehydration. With the exception of bile acids and albumine, parameters indicating liver and kidney functions (BUN, creatinine) were not significantly correlated with the endotoxin or PG-metabolite concentrations.

Symptoms previously linked with sublethal doses of endotoxin displayed by the pyometra bitches in Study IV and V respectively, were e.g. fever (60% and 50%), lethargy (90% and 85%), tachycardia (60% and 28%) and tachypnea (40% and 25%) (Van Miert & Frens, 1968). Other endotoxin-linked symptoms such as

anorexia and vomiting were recorded in 50% and 70% of the pyometra bitches in Study IV and V (Van Miert & Frens, 1968).

Mean values of selected biochemical and hematological blood parameters in Study V are presented in Table 3. The results from hematological and biochemical analyses of the pyometra groups in both Study IV and V generally confirm what previously has been shown in bitches with pyometra (Børresen, 1980; Fransson, 2003). The significantly increased α_2 -, β_1 - and γ -globulin fractions of serum proteins in the pyometra bitches compared with the controls (Study IV) are likely to reflect an increased synthesis of acute phase proteins and antibodies in response to the bacterial infection. Toxic effects on granulocytes and erythrocytes in seven (Study IV) and two (Study V) of the pyometra cases, respectively, confirm the influence of toxins. In 20% of the pyometra bitches in Study IV, bile acid concentrations were elevated, which could result from the intrahepatic cholestasis previously associated with pyometra in bitches (Børresen & Skrede, 1980). In pigs elevated bile acids (and PG-metabolite levels) are seen as a result of experimentally injected ET, explained by cell damage of the hepatocytes, prostaglandin-induced impaired liver function and metabolism and slow bile flow (Holst, Edqvist & Kindahl, 1993). Increased blood level of cholesterol, as confirmed in the pyometra bitches in both Study IV and V, is also a feature of intrahepatic cholestasis. Hypocalcemia, shown to be a result of endotoxin injections in pigs (Holst, Edqvist & Kindahl, 1993), goats (Fredriksson, Kindahl & Edqvist, 1984) and cattle (Aiumlamai & Kindahl, 1990) was, however, not associated with pyometra. Only mild hypocalcemia was detected in two of the pyometra bitches in Study V.

Based on the above mentioned findings, it may be possible that treatments tempering the release or effects of endotoxin (Fletcher & Ramwell, 1977; Odensvik & Magnusson, 1996), can be beneficial for bitches with pyometra.

... and with increased levels of prostaglandin F_{2α}

Of the 69 bitches included in Study V, 58 were diagnosed with pyometra and 11 with CEH. The results showed that the levels of PG-metabolite were significantly increased in bitches with pyometra compared with bitches with CEH in Study IV ($P=0.01$) and the controls in Study IV and V ($P<0.05$ and $P\leq 0.001$ respectively). The number of bitches in the different patient groups and controls in Study V, divided into five different categories of PG-metabolite levels, are illustrated in Fig. 4. Mean PG-metabolite levels, ranges and standard deviations for the different groups are displayed in Table 3. In Study IV the mean level of PG-metabolite in the bitches with pyometra was 9830 pmol l⁻¹.

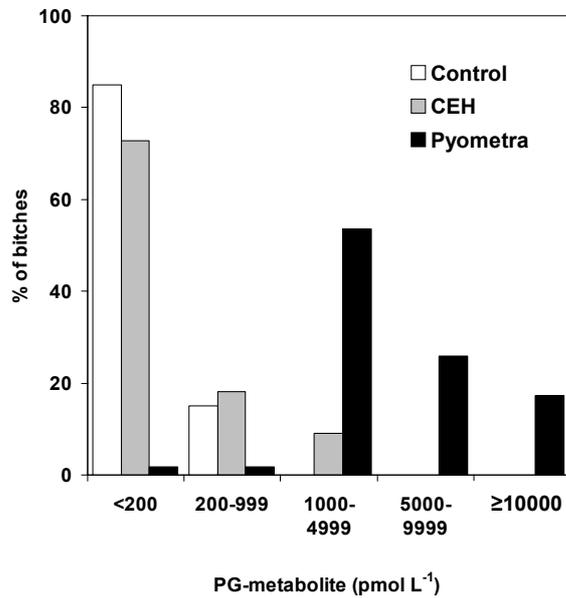


Fig. 4. Percentage of the controls, cystic endometrial hyperplasia (CEH) and pyometra bitches as divided into five groups according to their prostaglandin $F_{2\alpha}$ metabolite (PG-metabolite) plasma levels.

The increased concentrations of PG-metabolite in bitches with pyometra are most likely derived from endometrial synthesis of prostaglandins initiated by the uterine bacterial infection and subsequent endotoxin release, as is observed in many animal species (Heap & Poyser, 1975; Hughes *et al.*, 1979; Aiumlamai & Kindahl, 1990; Vandeplassche, Coryn & De Schepper, 1991; Mateus *et al.*, 2003). The levels of PG-metabolite were, however, not significantly higher in bitches with CEH compared with the controls. This can be explained by the lack of bacterial infection and endotoxemia in cases with CEH. Any inflammation present in the CEH group, as indicated by higher mean levels of PG-metabolite compared with the control group, was not significant (Table 3).

Table 3. Mean values and standard deviation (SD) for selected hematological and blood chemistry parameters in bitches with pyometra or CEH and healthy controls in Study V. Temperature=body temperature; PG-metabolite=prostaglandin F_{2α} metabolite; EVF=red blood cell volume fraction; WBC=white blood cell count; PBN=percentage band neutrophils; BN=band neutrophils; Neutrophils=segmented neutrophils; ALAT=alanine amino transferase; AP=alkaline phosphatase; BUN=blood urea nitrogen.

	Control		CEH		Pyometra	
	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
Temperature (°C)	38.7 ± 0.4	20	39.1 ± 0.7	8	39.1 ± 0.6	56
PG-metabolite (pmol l ⁻¹)	78 ± 208	20	377 ± 956	11	6278 ± 5757	58
Hemoglobin (g l ⁻¹)	173 ± 17.3	20	138 ± 19.4	11	136 ± 24.4	57
EVF (%)	0.50 ± 0.05	20	0.39 ± 0.05	11	0.38 ± 0.07	57
WBC (10 ⁹ l ⁻¹)	8.8 ± 2.6	20	10.6 ± 6.4	11	22.8 ± 11.4	56
BN (10 ⁹ l ⁻¹)	0.01 ± 0.02	20	0.55 ± 1.25	11	2.86 ± 2.96	56
PBN (%)	0.05 ± 0.22	20	3.00 ± 6.19	11	11.9 ± 9.97	56
Neutrophils (10 ⁹ l ⁻¹)	5.5 ± 1.7	20	7.8 ± 5.0	11	15.3 ± 8.1	56
Eosinophils (10 ⁹ l ⁻¹)	0.7 ± 0.6	20	0.3 ± 0.3	11	0.3 ± 0.6	56
Lymphocytes (10 ⁹ l ⁻¹)	2.1 ± 0.9	20	1.0 ± 0.5	11	1.5 ± 0.9	56
Monocytes (10 ⁹ l ⁻¹)	0.4 ± 0.2	20	0.9 ± 0.5	11	2.8±2.3	56
Platelet count (10 ⁹ l ⁻¹)	312 ± 102	20	250 ± 104	10	233 ± 118	55
Creatinine (μmol l ⁻¹)	86 ± 22	20	77 ± 29	9	81 ± 42	53
ALAT (μkat l ⁻¹)	0.6 ± 0.3	19	0.6 ± 0.4	10	0.6 ± 0.8	57
AP (μkat l ⁻¹)	1.7 ± 0.9	20	2.6 ± 2.3	9	6.0 ± 6.0	57
BUN (mmol l ⁻¹)	6.6 ± 2.9	20	4.6 ± 1.7	9	5.1 ± 4.0	48
Protein (g l ⁻¹)	79 ± 7	20	77 ± 6	9	80 ± 13	49
Albumin (g l ⁻¹)	34 ± 4	20	31 ± 3	9	27 ± 5	49
Cholesterol (mmol l ⁻¹)	7.4 ± 2.0	20	7.3 ± 2.6	9	9.8 ± 2.5	48
Calcium (mmol l ⁻¹)	2.7 ± 0.3	20	2.7 ± 0.2	9	2.6 ± 0.3	48

PG-metabolite was correlated to a large number of measured parameters in both Study IV and V, including Hb, WBC, BN, segmented neutrophils, monocytes and albumin. In addition, correlations were found between PG-metabolite and bile acids, albumine/globuline quota, γ -globulines and β -1 globuline fraction of serum protein electrophoresis in Study IV. In Study V, PG-metabolite levels were significantly correlated to EVF, PBN, lymphocytes, MCV, creatinine, ALAT, glucose, and cholesterol. PG-metabolite levels were also significantly correlated to clinical findings including duration of illness prior to admission to the clinic (according to information from the owner), length of the hospitalisation, the general clinical condition of the bitch, rectal temperature, heart rate and degree of dehydration at the time of admission.

As for prognostic value, the PG-metabolite concentrations were correlated to morbidity as measured by length of the hospitalisation and the general state of health of the bitch at admission (Study V). Bitches operated for pyometra are generally dismissed 1-2 days after surgery. Additional complications with severely depressed general condition will result in longer hospitalisation, a factor used for these estimations also in studies in man (Afessa *et al.*, 2001). The preoperative measurement of PG-metabolite can thus help the clinician in roughly determining the systemic severity of the pyometra.

To be able to evaluate the possible value of PG-metabolite in the prediction of survival or death, more patients are needed since only two bitches died of causes related to pyometra in Studies IV and V. Two other bitches died, 2 and 14 days after surgery respectively, but it is uncertain whether the deaths were consequences of the pyometra since no autopsies were performed. The bitch with the highest observed level of PG-metabolite, 33,500 pmol l⁻¹, died the day after surgery as a consequence of myocarditis and other sequela of disseminated bacterial infection. The other confirmed death was a bitch with a thin-walled uterus which ruptured during the operation. That bitch died one hour after the surgery was completed but had low (400 pmol l⁻¹) levels of PG-metabolite before surgery. This was perhaps an example of severely damaged or atrophic endometrium which reduces the endogenous production of prostaglandins, as has been described in the mare (Hughes *et al.*, 1979).

The levels of PG-metabolite were correlated to rectal temperature, heart rate, degree of dehydration, WBC and PBN. These are four out of five of the clinical criteria used to determine whether the systemic inflammatory response syndrome (SIRS) is present. The presence of SIRS has clinical importance since it has been linked with higher mortality (Brady & Otto, 2001; Okano *et al.*, 2002). When PG-metabolite levels in bitches with and without SIRS were compared, the differences were significant for the group consisting of both pyometras and CEH cases, just significant for the pyometra group but not significant for the CEH group (Fig. 5). PG-metabolite analysis could therefore be valuable in the detection of systemic inflammation if a rapid test for veterinary clinics becomes available in the future.

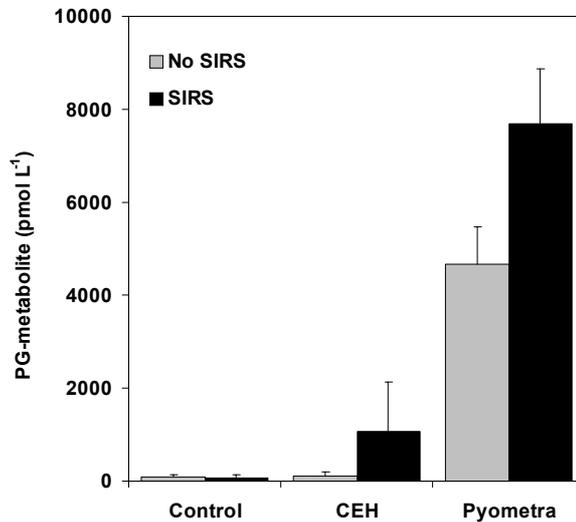


Fig. 5. Prostaglandin $F_{2\alpha}$ metabolite (PG-metabolite) concentrations in controls and bitches with cystic endometrial hyperplasia (CEH) or pyometra, with and without a systemic inflammatory response (SIRS). Bars indicate 1 standard error of the mean.

PG-metabolite distinguishes pyometra from CEH

The separation of bitches with pyometra from bitches with CEH can be clinically difficult. Fluid in the uterine lumen may be detected by diagnostic imaging in both conditions, and the clinical presentation, history and laboratory parameters are very similar (De Bosschere *et al.*, 2001; Fransson *et al.*, 2004). Bitches with pyometra should be regarded as emergencies since there is a risk of uterine rupture, endotoxic shock and rapid deterioration of the general status. In contrast, in bitches with CEH surgery can, if necessary, be postponed until optimal surgical conditions are available without further negative consequences for the bitch. Clinically it would therefore be helpful to accurately predict the diagnosis before surgery to optimise treatments.

Clinical examinations useful to differentiate between pyometra and CEH are the general attitude of the bitch at the time of admission, lethargy and gastrointestinal signs as confirmed in the present study (Table 4). These symptoms might reflect the presence of endotoxemia in pyometra as opposed to in CEH. The hematological and blood biochemical parameters that displayed significant differences between bitches with pyometra and CEH are shown in Table 5.

Table 4. The presence of selected abnormal case history and physical examination findings in bitches with pyometra or cystic endometrial hyperplasia (CEH) and significant differences between the two patient groups.

History / clinical finding	CEH (n=11)	Pyometra (n=58)	P-value (Fisher's exact test)
Gastro-intestinal signs	10% (10)	70% (56)	<0.001
Lethargy	10% (10)	64% (56)	0.004
Attitude \geq moderately depressed at admission	40% (10)	85% (55)	0.005

Table 5. Mean and standard deviations for selected hematological and blood biochemical parameters that were significantly different in bitches with pyometra compared with cystic endometrial hyperplasia. PG-metabolite = prostaglandin metabolite 15-keto-13,14-dihydro-PGF_{2 α} ; WBC = White blood cell count; PBN = percentage band neutrophils. Two-sample T-tests were used for the calculations of P-values.

	Pyometra		CEH		P-value
	Mean (\pm SD)	N	Mean (\pm SD)	N	
PG-metabolite (pmol l ⁻¹)	6278 (\pm 5757)	58	377 (\pm 956)	11	0.001
WBC (10 ⁹ l ⁻¹)	22.8 (\pm 11.4)	56	10.6 (\pm 6.4)	11	0.001
Band neutrophils (10 ⁹ l ⁻¹)	2.86 (\pm 2.96)	56	0.55 (\pm 1.25)	11	0.015
PBN (%)	11.9 (\pm 9.97)	56	3.00 (\pm 6.19)	11	0.001
Segmented neutrophils (10 ⁹ l ⁻¹)	15.3 (\pm 8.1)	56	7.8 (\pm 5.0)	11	0.006
Monocytes (10 ⁹ l ⁻¹)	2.8 (\pm 2.3)	56	0.9 (\pm 0.5)	11	0.010
Cholesterol (mmol l ⁻¹)	9.8 (\pm 2.5)	48	7.3 (\pm 2.6)	9	0.009
Albumin (g l ⁻¹)	27 (\pm 5)	49	31 (\pm 3)	9	0.026

PG-metabolite was the single parameter with highest sensitivity (96.6%) and specificity (90.9%) for the differentiation of pyometra versus CEH. This sensitivity is higher than what has been reported for any single parameter previously in this differentiation (Fransson *et al.*, 2004). Routine blood parameters such as WBC, BN, monocytes, albumin, and ALP have low diagnostic value because of low specificity ($\leq 18.1\%$). Combining PG-metabolite with each of these parameters led to decreased specificity as compared with PG-metabolite alone. The sensitivity also slightly decreased, except for the combination of PG-metabolite and WBC, which yielded a sensitivity of 98.2% (specificity 81.8%). In clinical work it is preferred to have a highly sensitive test since the surgery on a “false positive pyometra” (CEH) patient is less risky compared with a pyometra falsely diagnosed as CEH and perhaps not monitored and treated as an emergency. An absolute identification of pyometra versus CEH was obtained when combining the analysis of PG-metabolite with PBN, which yielded a sensitivity of 100.0% and a specificity of 81.8%. The equation used for calculation of the estimated probability the diagnosis pyometra versus CEH for PG-metabolite in combination with PBN (as shown in Fig. 6) was as follows:

$$\text{Estimated probability of pyometra} = \frac{\exp(-0.9684 + (0.001082) \cdot \text{PG-metabolite} + (0.077413) \cdot \text{PBN})}{1 + \exp(-0.9684 + (0.001082) \cdot \text{PG-metabolite} + (0.077413) \cdot \text{PBN})}. P \leq 0.0001.$$

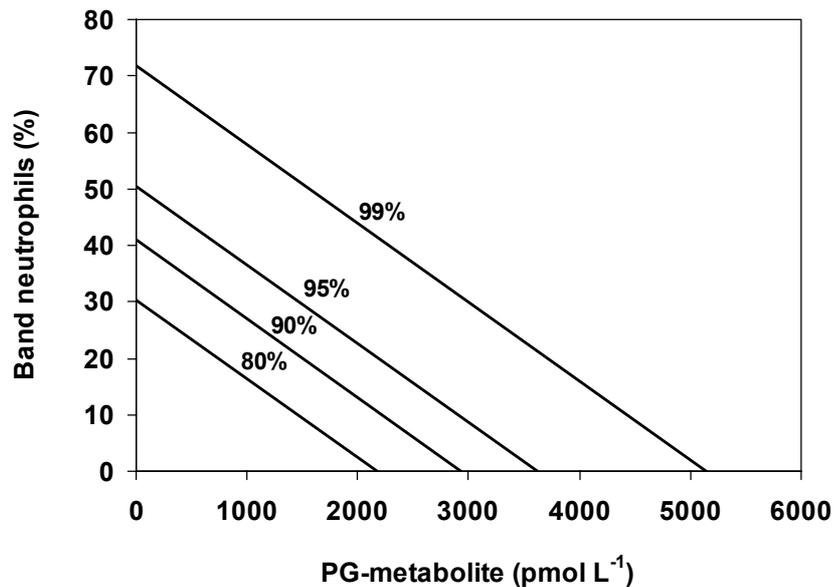


Fig. 6. Contour plot of estimated 80%, 90%, 95% and 99% probability of pyometra versus cystic endometrial hyperplasia (CEH) in a bitch based on percentage band neutrophils (PBN) and plasma prostaglandin F_{2α} metabolite (PG-metabolite) concentrations. Bisection of the values for PBN and PG-metabolite to the right of each respective graph indicates 80%, 90%, 95% or 99% probability of the diagnosis pyometra.

Furthermore, if PG-metabolite was used alone and if the PG-metabolite level in a bitch is 4734 pmol l⁻¹, there is a 99% probability of the diagnosis pyometra versus CEH. PG-metabolite levels of 3232 pmol l⁻¹, 2552 pmol l⁻¹ and of 1815 pmol l⁻¹ indicate a 95%, 90% or 80% probability of pyometra, respectively. The formula obtained in this case was:

Estimated probability of pyometra = $\exp(-0.60784+0.001099*\text{PG-metabolite}) / (1+ \exp(-0.60784+0.001099*\text{PG-metabolite}))$. P<0.0001.

The analysis of PG-metabolite concentrations therefore has high diagnostic value. At high PG-metabolite levels (above about 3000 pmol l⁻¹), PG-metabolite alone is enough for differentiation of pyometra from CEH, but at lower PG-metabolite levels (about 2000 pmol l⁻¹) the combination of PG-metabolite with PBN, WBC or BN (if sufficiently high) will increase the probability of detection of pyometra (formulas regarding WBC and BN not shown).

Conclusions

The results presented in this thesis yield the following conclusions:

- There are clear age- and breed-related differences in the incidence of pyometra in bitches in Sweden, which should be taken into account when studying the disease. It is probable that some breeds carry a higher genetic predisposition for pyometra than other breeds. Given the different age patterns for risk, the maximum or optimal age for elective spaying to prevent pyometra differs among breeds.
- Uterine *E. coli* isolates from different bitches with pyometra are genetically different. Pyometra is thus not caused by one or a few bacterial clones spread between animals but more likely by bacteria with origin in the normal flora of each bitch. Genetically similar *E. coli* strains from one pyometra uterus can exhibit different colony morphology. In cases of *E. coli* pyometra with a concurrent subclinical urinary tract infection, the urinary tract and the uterus are likely to be infected with the same bacterial strain.
- Antimicrobial resistance is not a likely cause for recurrence after medical treatment of *E. coli* pyometra in Sweden. Data on antimicrobial susceptibility of *E. coli* from urinary tract infections is not suitable as basis for antimicrobial drug selection in treatment of *E. coli* pyometra.
- Bitches with pyometra have increased plasma levels of endotoxin.
- Plasma concentrations of the PG-metabolite provide a good indicator of endotoxin release in bitches with pyometra. Other hematological and blood biochemical parameters were not as useful as PG-metabolite for that purpose.
- PG-metabolite analysis is useful in the differentiation between pyometra and CEH.
- PG-metabolite analysis is also valuable for the prediction of increased morbidity in pyometra, as measured by hospitalisation length or presence of the systemic inflammatory response syndrome (SIRS).

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Populärvetenskaplig sammanfattning

Pyometra, eller varbildande livmoderinflammation, är en vanlig sjukdom hos hundar som ger upphov till symtom från reproduktionsorganen och många andra av kroppens organsystem. Sjukdomen är dödlig om den inte behandlas, och vissa tikar är så allvarligt allmänpåverkade att de inte överlever, trots behandling. Syftet med denna avhandling var att undersöka epidemiologiska, bakteriella och inflammatoriska aspekter av sjukdomsutvecklingen vid pyometra. Ökad kunskap inom dessa områden kan komma att leda till optimerade behandlingsrutiner och ökade möjligheter att på sikt minska eller förhindra uppkomsten av sjukdomen.

Uppgifter från hundar försäkrade i försäkringsbolaget Agria användes till att undersöka skillnader i förekomsten av pyometra med avseende på ålder och ras. Av de tikar som ingick i studien drabbades i genomsnitt 23-24 % av pyometra före 10 års ålder. Andelen varierade mellan 10 % och 54 % beroende på ras. Det finns således viktiga ras- och åldersrelaterade skillnader i förekomsten av pyometra. Raser med en hög risk att utveckla pyometra är långhårig collie, rottweiler, cavalier king charles spaniel, golden retriever, berner sennenhund och engelsk cocker spaniel. Raser med låg risk att utveckla sjukdomen är drever, schäfer, tax (miniatyr och normalstor) och hamiltonstövare.

För att utvärdera om de bakteriekloner som orsakar sjukdomen är nära besläktade utfördes en genotypisk undersökning av *Escherichia coli* (*E. coli*), den bakterie som vanligen isoleras vid pyometra. Resultaten visar att infektionen inte orsakas av en eller ett fåtal bakteriekloner. Bakteriestammarna uppvisar en stor variation vilket tyder på att ursprunget är kopplat till tikens normala bakterieflora. Alla bakterieisolat från en och samma livmoder är dock av samma genotyp. I fall av pyometra och samtidig subklinisk urinvägsinfektion, är troligen både livmoder och urinvägar infekterade med samma bakteriestam.

Vid antibiotikabehandling av tikar med pyometra grundar sig valet av preparat oftast på studier gjorda i andra länder och främst gällande *E. coli* från urinvägsinfektioner. I denna studie undersöktes den antimikrobiella känsligheten hos *E. coli* för de i hundpraktik mest använda antibiotikapreparaten. Bakteriestammarna var isolat från både tikar med pyometra och hundar med urinvägsinfektioner. Dessutom jämfördes dessa data med motsvarande resultat från en liknande samling med 10 år äldre data för att avgöra om någon skillnad i resistensutveckling kunde påvisas. I regel var resistensen för antimikrobiella preparat låg, och inga större förändringar påvisades under en tioårsperiod. Hos bakterieisolat från pyometra var känsligheten högre för ampicillin, streptomycin och tetracykliner jämfört med urinvägsisolat inskickade från djur som behandlats på djursjukhus. Det var dock ingen skillnad när urinvägsisolat från hundar som behandlats på mindre djurkliniker jämfördes med pyometraisolat. Generellt sett bör således inte resultat från undersökningar av *E. coli* från urinvägsinfektioner användas som bas för val av antibiotika vid behandling av pyometra.

Förekomst och inverkan av endotoxiner från Gram-negativa bakterier som *E. coli* vid sjukdomsutvecklingen av pyometra hos hundar undersöktes i en klinisk studie. Hos andra djurslag har koncentrationer av prostaglandin $F_{2\alpha}$ (som mäts genom sin metabolit 15-keto-13,14-dihydro-PGF $_{2\alpha}$) (PG-metabolit) visat sig vara en känslig indikator för endotoxin i blodet och frisätts dessutom vid inflammationer i livmodern. Därför utvärderades om koncentrationen endotoxin i blodet var korrelerad med PG-metabolitnivån även hos tikar med pyometra. Hur halten av endotoxiner relaterar till andra blodbilds- och biokemiska analyser undersöktes också. Resultaten bekräftar att tikar med pyometra hade förhöjda nivåer av endotoxin och PG-metabolit i blodet. PG-metabolit kan vara en indikator för graden av endotoxinpåverkan hos hundar med pyometra eftersom dess nivåer var signifikant korrelerade med halten av endotoxin och analysen är jämförelsevis pålitlig och kostnadseffektiv.

Koncentrationer av PG-metabolit i blodet hos tikar med pyometra eller cystisk endometrie hyperplasi (CEH)/mucometra undersöktes för att avgöra om nivåerna kan skilja de båda sjukdomstillstånden åt. Vidare utfördes en rad biokemiska och blodbildsanalyser samt bedömningar om kliniskt status hos patienterna. Tikar med pyometra hade signifikant högre nivåer av PG-metabolit i blodet jämfört med tikar med CEH och friska kontrollhundar. Som ensam analys hade PG-metabolit en god förmåga (sensitivitet 96.6% och specificitet 90.6%), att identifiera om en tik led av pyometra eller CEH. Om både analys av procent stavkärniga neutrofila granulocyter och PG-metabolitnivåer användes kunde samtliga tikar med pyometra skiljas från de med CEH (sensitivitet 100 % och specificitet 81,8 %). Analys av PG-metabolit kan dessutom användas som ett mått på hur allvarligt sjukdomstillståndet är hos en tik med pyometra. PG-metabolitnivåerna var korrelerade både till antal dagar patienten fick stanna på djursjukhuset efter operation och till förekomsten av ett systemisk inflammationssvar (vilket vid andra sjukdomar har kopplats till högre dödlighet).

Sammanfattningsvis bidrar resultaten av denna avhandling med data om ras- och åldersrelaterade skillnader vid förekomsten av pyometra, vilket kan vara till stor nytta för framtida forskning och tänkbara avelsprogram. Dessutom framkommer flera viktiga bakteriologiska och patofysiologiska fakta om sjukdomsutvecklingen. Dessa fakta kan i sin tur leda till en förbättrad diagnostik samt effektivare behandlingsstrategier för tikar med pyometra som därmed minskar lidandet och ökar överlevnadsfrekvensen.