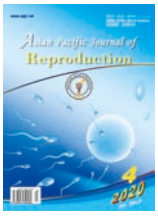

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Prospects of diagnostic and prognostic biomarkers of pyometra in canine

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ABSTRACT

Pyometra is one of the most common uterine pathologies of intact bitch at middle to advanced age. In the early stages, the disease shows subtle changes, making diagnosis a challenge. In contrast, at later stages, it manifests as potentially life-threatening systemic inflammatory response syndrome. Ultrasonographic examination of the uterus aids in the diagnosis, although it has limitation in ascertaining the clinical severity of pyometra. Moreover, differentiation of cystic endometrial hyperplasia from pyometra could not be discerned with greater accuracy. Therefore, false negative diagnosis of pyometra patients leads to development of systemic inflammatory response, which delays administration of therapies and results in deaths during early course of treatment. Further, indiscriminate use of broad-spectrum antimicrobials at higher dose in false positive cases considerably contributes to the rising pool of drug resistant pathogens, thereby increasing the risk of case fatality due to sepsis in a long-term. Monitoring the circulating pro-inflammatory cytokines, acute phase proteins, endotoxin, growth factors and inflammatory mediators is the current trend in pyometra research, especially for developing diagnostic and prognostic biomarkers. The present review deals with the prospects of developing diagnostic and prognostic biomarkers in the canine pyometra.

KEYWORDS: Pyometra; Systemic inflammatory response; Acute-phase proteins; Prostaglandin F metabolite; Canine

1. Introduction

In India, the pet population has grown from 7 million in 2006 to 10 million in 2011. On an average, 0.6 million pets are adopted every year. The Indian pet market is now $800 million plus industry, and is expected to register strong double-digit retail value growth in the coming years. The socio-cultural sensitization towards pets is changing; more pet owners have come to humanize their pets, and do not mind spending on what they perceive as being necessary or beneficial for their pets. With increasing awareness about the good health and well being of the pet, pet healthcare industry is also expanding. Pet owners are ready to spend handsome amount for good health of their pets and routine vet visits are in trend be it for pet food, medicines, vaccines or diagnostic tests[1]. The pet owners prefer female over male for ease of management and compatibility. Apart from registered kennel club, majority of owners in urban and peri-urban areas rear pet without spaying or breeding[1].

Among the reproductive tract pathologies, uterine diseases are the common cause of infertility. Middle to aged nulliparous bitches or those frequently exposed to hormonal therapy are at high risk of developing cystic endometrial hyperplasia, pyometra and neoplasia of the uterus, ovaries and mammary glands. Among these, pyometra is the most prevalent and late diagnosed condition. It is characterized by persistent endometrial inflammation and bacterial colonization, consequent with accumulation of endotoxin and establishment of life threatening complications as systemic inflammatory response syndrome (SIRS)[2]. SIRS is determined on the base of certain vital clinical parameters such as pulse and respiratory rates, rectal temperature and total blood leukocyte concentration[2]. Rapid

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diagnosis of pyometra and differentiation from cystic endometrial hyperplasia in bitches are essential for adopting effective treatment strategies. The prognosis is poor to grave, if the diagnosis is not made at the early stage, especially in closed cervix pyometra. It has been reported that more than 50% bitches with SIRS positive pyometra are associated with poor prognosis and longer period of hospitalization[3]. Although conservative medical treatment can be used in selected cases, ovariohysterectomy is the safe and most effective treatment of pyometra[4,5]. Developing diagnostic biomarkers, therefore, is the need of the hour for timely initiation of treatment to increase chance of animal survival and quality life. This review describes the recent findings on the potential biomarkers on the diagnosis and prognosis of canine pyometra.

2. Incidence

After passing recurrent oestrous cycles, mature bitches develop pyometra at an average 7.25 years of age[6]. Generally, the clinical signs of pyometra occur 15-20 days after oestrus but may also appear at proestrus, post-mating or even at anestrus stage[7]. The incidence of pyometra goes up to 20% in different breeds of the bitches at a median age of 10 years[8]. Reports show that the nulliparous bitches contribute nearly 75% cases of canine pyometra[9]. Based on the registered clinical cases at a referral veterinary polyclinic, we reported that the point prevalence of canine pyometra was 28%[10].

Breed and genetic factors strongly predispose to the development of pyometra[11]. In India, the breeds at high risk of development of pyometra include: Labrador, Spitz, German Shepherd, and Dalmatian; however, Doberman, Dachshund, Great Dane, Pug, Boxer, Lhasa Apso, Cocker Spaniel, Saint Bernard, English Bulldog and Neapolitan Mastiff show lower risk of pyometra[12–14]. In a recent study based on hospital occurrence, it has been reported that the incidence of pyometra is highest in the Labrador (28.89%), followed by Spitz (22.22%), non-descript (20.00%) and German Shepherd (8.89%). The incidence of pyometra is relatively lower in the Doberman, Pug, Saint Bernard, Rottweiler and Dalmatian breeds[10].

3. Aetiopathology

In the common dogma, cystic endometrial hyperplasia induced endometrial degeneration along with cystic distension of endometrial glands and fibrotic changes provide favourable conditions for the establishment of bacterial colonization. The compromised endometrial epithelium possesses optimal adhesion factors that promote opportunistic pathogens from the vagina to proliferate and establish the infection[15]. This school of thought suggests that cystic endometrial hyperplasia usually develops prior to pyometra development; however, it is also noticed that cystic endometrial hyperplasia does not inexorably progress to pyometra. This is obvious, as all dogs develop some degree of cystic endometrial hyperplasia in advanced age, whereas only a subset of bitches develops pyometra. Moreover, pyometra is reported even in young bitches without any clinical or pathological evidence of cystic endometrial hyperplasia[16].

Polycystic ovaries and active ovarian neoplasia cause imbalance between estrogen and progesterone resulting into prolonged estrus. Repeated exposure of the uterus to sustained and high progesterone concentration drive the development of endometritis-pyometra complex[17]. Progesterone stimulates proliferation, coiling and secretion of the glandular endometrium with concomitant promotion of myometrial quiescence and closer of cervix that provides a favourable micromillieu for the growth and colonization of opportunistic pathogens that ascend from the vaginal flora[7]. The effects are cumulative, with each oestrous cycle aggravating the uterine pathology, support the greater incidence of pyometra in middle to advanced aged bitches. Suppression of innate immunity is observed in the first part of diestrus due to higher progesterone concentration preceded by minimal estrogen release[18] and the local production of progesterone might also contribute to the pathogenesis of pyometra. Although circulating progesterone concentration remains within the normal range to influence the development of cystic endometrial hyperplasia, Gultiken *et al*[19] recently suggested that expression of 3β-hydroxysteroid dehydrogenase, the terminal enzyme in the progesterone biosynthesis, is upregulated in the endometrium of bitches with the cystic endometrial hyperplasia-pyometra complex.

Escherichia coli was isolated in 62% to 90% pyometra cases[10], as it is part of the vaginal flora that enter into the uterus during the proestrus and estrus or after subclinical urinary tract infection[20]. *Escherichia coli* isolates in the pyometra are invariably uropathogenic strains and share the common virulence factor genes[21]. Other bacterial species such as *Streptococcus* spp., *Staphylococcus* spp., *Proteus* spp., *Klebsiella* spp., *Corynebacterium* spp., *Pseudomonas* spp., *Pasteurella* spp., *Enterococcus* spp., *Serratia* spp., *Bacillus* spp. and *Haemophillus* spp. are also reported in cases with pyometra[10].

4. Clinical signs

A typical bitch with pyometra is usually presented at diestrus, with a history of genital discharge and/or systemic signs of diseases. Clinical signs depend on the accumulation of pus, which, in turn depends on the degree of cervical patency. The onset of clinical signs may be very acute or steady and often show more severity with closed cervix pyometra[7]. A purulent vaginal discharge is frequently present if the cervix is open, and the systemic signs include polydipsia, polyuria, anorexia, dehydration, vomiting, diarrhea, abdominal pain, lethargy, fever or hypothermia, tachycardia and tachypnea[3]. Except intermittent vaginal discharge, clinical signs

are less conspicuous in open-cervix pyometra. In contrast, dogs with closed cervix pyometra are more serious, severely dehydrated, toxemic and easily develop SIRS.

5. Diagnosis

Pyometra is regarded as the most severe end-stage of the gradually developing CEH–pyometra complex, although it has been suggested that the disease could be divided into, CEH/mucometra and endometritis/pyometra, depending on the clinical signs and the induced inflammatory response[6,16]. In CEH, the character of the intrauterine fluid differs from pyometra, in that it is classically all sterile and the fluid is seromucous, bloody, or serous, while pyometra presents infected purulent fluid in the uterine cavity. It is easy to diagnose open cervix pyometra; however, it is challenging in bitches with closed cervix pyometra when the history and clinical picture are incomprehensible.

In the absence of systemic signs, a diestrus bitch with purulent vaginal discharge should be suspected for pyometra[16]. A tentative diagnosis is based on the anamnesis, clinical examination and complete blood count. Ultrasonography provides confirmatory diagnosis[22] and the typical findings include endometrial thickening with or without cystic changes, distended and sacculated uterus with an accumulation of anechoic fluid with small echogenic particles[22,23], occasionally filled with gasses[24]. The inflammatory response is more pronounced in pyometra compared with CEH/mucometra as reflected by increased circulating concentration of C-reactive protein (CRP), prostaglandin F metabolite (PGFM) and several other laboratory parameters[3,5,9].

As pyometra frequently culminates in life threatening SIRS, timely diagnosis is crucial for the clinical management. Diagnosis of pyometra led SIRS is now practiced based on certain vital clinical parameters such as pulse and respiratory rates, rectal temperature and complete blood count in circulation[3]. The accuracy of these parameters, however, are low resulting in false-positive or false-negative diagnosis ultimately leading to increased mortality rate due to delay in the treatment. Besides, frequent use of broad-spectrum antimicrobials in false-positive SIRS cases contributes substantially to the pool of drug-resistant pathogens and increases the risk of death due in a long-term[25]. The blood concentrations of prostaglandin metabolites, acute-phase proteins and CRP are increased in SIRS-positive pyometra patients *vs.* SIRS-negative pyometra patients, and the levels are associated with morbidity as measured by the length of hospitalization[3,11]. The other uterine pathologies like endometritis and uterine neoplasia are less commonly diagnosed in clinical practice.

5.1. Effect on haemato–biochemical parameters

A marked alteration in the haematology and serum biochemistry is noted in the canine pyometra[26]. Leukocytosis with increased band neutrophils, monocytosis, toxic degeneration of neutrophils, anaemia are observed in bitches with pyometra[27]. A normocytic, normochromic anaemia is considered as an indicator of chronic pyometra when erythropoiesis is compromised because of the toxic effects of uterine fluid on the bone marrow, lack of available iron and diapedesis of erythrocytes to the uterus[28].

Besides the hematopoietic system, other vital organ functions including kidney and liver are likely to be altered in response to endotoxin and inflammatory mediators. In pyometra, increased serum concentrations of creatinine, blood urea nitrogen, hypoalbuminemia, hyper gamma-globulinaemia and proteinuria have been reported[29]. The impaired renal function occurs due to the inflammation of tubulo-interstitial area or immune-complex associated glomerulonephritis resulting in urinary dysfunction[26]. Moreover, endotoxaemia with pyometra can cause intrahepatic cholestasis and alteration of hepatocellular function. The major enzymes of the hepato-biliary system such as alanine aminotransferase and aspartate aminotransferase are altered depending on the degree of severity in hepatocellular damage associated with pyometra[10]. An increased salivary adenosine deaminase activity (ADA) is noted in the dogs with SIRS positive pyometra than the control[30]. Moreover, the salivary ADA had a moderate positive correlation with leukocyte and band neutrophil counts, haptoglobin, salivary α-amylase and a low positive correlation with CRP. Recently, concentration of serum and salivary adiponectin is reported to be low in the affected with septic pyometra as compared to healthy bitch, however the adiponectin concentration in saliva is tended to increase during post surgery period indicating its diagnostic importance in septic pyometra[31].

5.2. Endometrial transcripts profiling

Gene expression profile in the endometrium of cystic endometrial hyperplasia-pyometra positive bitches indicated marked upregulation of chemokines, cytokines, complement system and innate immune genes[32]. A key feature in the pyometra uterus was the increased expression of a battery of proteases, particularly matrix metalloproteinase, secretory leukocyte peptidase inhibitor (SLPI), prostaglandin synthase enzymes, cyclooxygenase-2 (COX2) and calprotectins of S100 family, namely S100A8 and S100A9[32,33]. Among the cytokine populations, a marked elevation of interleukin (IL)-1β, IL-6, IL-8, IL-10, 1L-15, 1L-18 and tumor necrosis factor α (TNF-α) was documented in the bitches with pyometra[34]. Further, a distinct up-regulation in expression of IL-6, IL-8, COX2 and prostaglandin F synthase (PGFS) was observed in the endometrium of pyometra positive bitches, especially in the more severe cases with endometrial atrophy[35]. In a recent study, it was recorded that the expression of IL-1β, IL-6, IL-8, COX-2, PGFS, and SLPI transcripts in bitches with pyometra was more prominent than that of the control. In cystic endometrial hyperplasia-pyometra, the IL-6 was significantly up-regulated when compared with

pyometra with no cystic endometrial hyperplasia, whereas S100A8 was higher in pyometra without cystic endometrial hyperplasia. Thus, the authors opined that measurement of IL-6 and S100A8 could serve as the potential diagnostic biomarkers to differentiate cystic endometrial hyperplasia from pyometra[36].

6. Prognostic indicators

Although pyometra is a critical life-threatening disease with a case fatality rate upto 10% including the cases managed by euthanasia, the prognosis is favourable in early diagnosed cases[11]. Pathologies such as polycystic ovaries, extensive cystic endometrial hyperplasia and senility are associated with poor prognosis even after aggressive medical treatment[37]. The prognosis is unfavourable when the case is complicated with secondary systemic infections. High mortality rates are observed in those cases that develop peritonitis[11]. Myocardial injury secondary to inflammation, disseminated bacterial infection or infarctions and endotoxaemia are suspected to be the potential contributing factors in unanticipated deaths[38]. Renal failure and end-stage cardiac dysfunction also contribute to the death in pyometra cases[39]. The prognosis is grave when anaemia is recurrent and blood coagulation process is severely compromised from disseminated intravascular coagulation along with thrombocytopenia[40].

6.1. Cytokines as prognostic indicator in pyometra led sepsis

Although it is well established that IL-1β, IL-6, IL-7, IL-8, IL-10, and TNF-α are locally upregulated in bitches with pyometra and SIRS, the information on the concentrations of these cytokines in the peripheral circulation with different stages of pyometra is limited. Of these, IL-8 and IL-10 are most extensively studied chemokines in the dogs[41]. A local and systemic elevation of IL-8 concentration has been reported in several inflammatory diseases[42]. IL-8 is a good predictor for the clinical characteristics of pyometra and can complement total leucocyte counts, neutrophil counts and CRP levels[43]. In dogs, IL-6 is one of the major pro-inflammatory cytokines associated with acute phase protein biosynthesis and glycosylation in the liver[44] and a high concentration of this cytokine reflects the initiation of inflammatory response in pyometra[45]. In contrast, the IL-10 has been recognized as a major suppressor of the immune response as it regulates the cytotoxic effects of monocytes and macrophages as well as the production of pro-inflammatory cytokines and acute phase proteins[46]. Serum IL-8 concentration was found to be greater in bitches with mild symptoms of pyometra than the control and dogs with severe symptoms. The analysis of serum IL-8 in canine pyometra showed an elevated concentration in dogs with moderate pyometra compared to dogs in a severe state of the disease as it plays a protective role in this disease[43]. Low concentrations of serum IL-8 in severe cases may be part of a well-orchestrated inflammatory response or the consequence of a depleted immune system[47]. A recent report showed the upregulation of *IL-6* and *IL-8* mRNA in the endometrium of hyperplastic pyometra and atrophic pyometra as compared to the control bitches[35]. Keratinocyte like chemokine, a novel chemokine, showed a significant increase in the dogs with pyometra led sepsis compared to those without sepsis (757 *vs.* 304 pg/mL). The concentration of KC-like chemokine was positively correlated with CRP, duration of hospitalization days, concentrations of IL-8, and percentage of monocytes and band neutrophils in bitches affected with pyometra[48].

6.2. Inflammatory mediators in circulation

Excess release of proinflammatory cytokines in response to bacterial infection in the uterus triggers the hepatic production of acute phase proteins[49]. Various studies suggested acute phase proteins are consistent and thus clinically valuable inflammatory indicators in dogs with pyometra[25,50] can be used to differentiate pyometra from mucometra/hydrometra[51]. In bitches, two major acute-phase proteins *viz*., CRP and serum amyloid A (SAA) are observed in clinical studies[52-54]. A greater concentration of CRP is observed in SIRS positive bitches as compared to SIRS-negative indicating its potential as putative marker of pyometra with sepsis[55]. Earlier studies also reported that circulatory SAA concentration increased during inflammation in the dogs[25]. The SAA concentration was significantly higher in bitches with pyometra than that of the control bitches (119.9 μg/mL *vs.* 10.0 μg/mL)[55].

6.3. Prostaglandin F metabolite

During inflammation prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$) is synthesized in the endometrium[56]. The concentration of $PGF_{2\alpha}$ is detected by measuring its metabolite 15-keto-(13,14)-dihydro-$PGF_{2\alpha}$ in the peripheral circulation[56]. Concentration of this metabolite increases in the bitches with pyometra and reduces after ovariohysterectomy. In pyometra, the estimation of plasma prostaglandin metabolite concentrations is vital for differentiation of cystic endometrial hyperplasia from pyometra for assessing the prognostic outcome in dogs[57]. Further, Hagman *et al*[57] reported significantly elevated plasma concentrations of endotoxin (49.0 pg/mL) and prostaglandin metabolite (24.7 nmoL/L) in pyometra and indicated the usefulness of prostaglandin metabolite as an indicator of endotoxin release. It has been observed that serum prostaglandin metabolite concentration of the pyometra positive bitches is predisposed by degree of systemic involvement and cervical patency. The SIRS positive bitches had significantly higher prostaglandin metabolite [(6.83±0.70) ng/mL *vs.*(4.12±0.40) ng/mL] than SIRS negative (P<0.05)[10].

6.4. Insulin-like growth factor I (IGF-I) and chromogranin A

IGF-I is produced primarily by the liver in response to the

stimulation by growth hormone and plays a significant role in cell proliferation, autocrine and paracrine activities including cystic endometrial hyperplasia-pyometra[58]. In the endometrium of bitches, the concentration of IGF-I depends on estrogen and progesterone[59]. Therefore, IGF-I is expected to be the highest in proestrus and oestrus. Due to its high mitogenic activity, IGF-I is considered as one of the key factors that mediate the expansion of endometrial lesions, including cystic endometrial hyperplasia[60]. It has been reported that activation of IGF-I receptor results in mobilisation of intracellular pathways of mitogenic signal transmission followed by increased expression of mRNAs and activation of cell proliferation[61]. However, the serum IGF-I concentration was reported to be lesser in pyometra positive bitches than that of the control bitches[55]. Decreased circulating concentration of IGF-I during inflammation is possibly due to the action of pro-inflammatory cytokines on its expression in the liver. Dabrowski *et al*[62] suggested that serum IGF-1 decreases and CRP level increases during pyometra; however, after ovariohysterectomy, IGF-1 is increased and CRP is decreased in the bitch.

Recently, the derivatives of chromogranin A are evaluated for its prognostic value for SIRS in critically ill human patients[63]. The concentration of catestin, but not vasostatin, showed a decrease in the bitches with pyometra as compared to the control[64]. Further, studies are warranted to appraise the potential diagnostic or prognostic values of these peptides in veterinary clinical conditions specially pyometra.

7. Treatment

The effective treatment of pyometra is ovariohysterectomy as it removes discomfort from the distended uterus and sources of endotoxin[65]. Anesthesia and surgery are hazardous in the bitches with severe disease[66]. In the absence of life threatening signs, pyometra can be treated medically in valuable bitches to restore fertility[67]. Prostaglandin and dopamine agonist are commonly used in the medical management of pyometra[11,68,69] as inhibition of progesterone biosynthesis is a prerequisite[66]. The combination of cabergoline at 5 μg/kg orally and a lower dose of synthetic prostaglandin at 5 μg/Kg subcutaneously for 7 days are the choice of drugs in the treatment of pyometra[10,69,70]. The combination of cloprostenol and cabergoline showed a recovery rate of 83%[68] and 51.43%[10], respectively. Prostaglandin is contraindicated in closed pyometra due to the risk of uterine rupture and fatal peritonitis.

In recent studies, combination of antiprogestin (aglepristone and mifepristone) and cloprostenol is reported to be effective for the treatment of open and closed cervix pyometra[71,10] and restored subsequent fertility[67]. The antiprogestin as a single drug is partially ineffective, as it blocks the progesterone receptors but does not exert any uterine contractile effect required for evacuation of uterine contents[16]. The subcutaneous administration of aglepristone 10 mg/kg on days 1, 2 and 8 alone with or without cloprostenol 1 μg/kg for 5 days (days 3 to 7) resulted in a recovery rate from 60% (aglepristone alone) to 84% on day 90 post-treatment[71]. Mifepristone has an advantage of oral medication compared to parenteral administration of aglepristone. The combined protocol of mifepristone 2.5 mg/kg orally (day 0, 1 and 10) and cloprostenol 5 μg/kg subcutaneously on alternate days for 7 days showed 85.7% non-recurrence rate of pyometra on day 10 post-treatment[10]. The prognosis for medical treatment of pyometra is more favorable in the absence of cystic endometrial hyperplasia and ovarian cysts[72] and in younger bitches[37]. However, there are chances of recurrence as the source of infection is not removed. Further, the standardization of endoscopic transcervical biopsy will help in early diagnosis[73,74]. Additionally, it is necessary to explore the drugs that counteract the progression of pyometra by minimizing the effects of hormone during early stage and late stage of clinical infection.

8. Conclusions

Despite the fact that pyometra is a disease of intact bitch at diestrus, the relative contributions of hormonal and infectious causes in the pathophysiology remains obscure. Current trends in the pyometra research shows promising development in serum and endometrial transcript based biomarkers for early diagnosis of pyometra and differentiation from cystic endometrial hyperplasia in bitches. The prognosis is poor if the diagnosis of pyometra is not made at the early stage, especially in closed cervix pyometra. Thus, diagnostic markers with high sensitivity and specificity are warranted to improve the treatment outcome in pyometra and to prevent the progression of SIRS into more severe multi-organ dysfunction and death. A reliable blood based diagnostic marker would be beneficial for diagnosing pyometra. It is worth emphasizing that the use of multiple markers may increase the likelihood of diagnosing pyometra over a solitary marker.

Conflict of interest statement

The authors have no conflict of interests to declare.

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Authors' contributions

Laishram Kipjen Singh, Manas Kumar Patra, Harendra Kumar and Krishnaswamy Narayanan conceptualized and designed the article. Girish Kumar Mishra, Abhishek Chandra Saxena, Ujjwal Kumar De and Sanjay Kumar Singh helped in designing the article. Laishram Kipjen Singh, Manas Kumar Patra and Krishnaswamy Narayanan prepared and revised the manuscript.

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