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Current Prospectives on histopathological and microbiological aspect of Pyometra: A Review

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Abstract
Pyometra is a potentially life-threatening disease of middle to older aged intact female animals due to various risk factors especially hormonal and microbial factors. Accumulation of the pus in the uterus after a period of progesterone dominance is usually observed in this disease condition. It is well established that the Cystic Endometrial Hyperplasia (CEH), a subclinical endocrine disease, usually precedes pyometra. But pyometra can develop independently of CEH. In this review, the role of various pathological and microbiological factors involved in the development and progress of pyometra will be discussed along with latest diagnostic methods for early recognition, diagnosis and therapeutic intervention.

Keywords: Pyometra, histopathology, microbiota, immunology, new generation sequencing, molecular markers

1. Introduction
Pyometra is one of the most common and important reproductive disorder of the female animals especially in unneutered bitches. More than 19% of intact female animals of 9-10 years age are usually affected by this disease condition [1, 2]. It requires costly medical and surgical involvement to cure and also can lead to many complications like sepsis, dissemination of microbes, peritonitis and multi-organ failure. The incidence of pyometra may vary from 2 to 55% with high occurrence in less than 10 years of age and 75-75% in nulliparous bitches [2]. A mortality rate of 1-10% is reported in bitches due to pyometra. The disease is usually presented with uterine infection with both local and systemic clinical manifestations that can be life-threatening if not treated timely due to septicemia and toxemia. Even though, the exact mechanism of pathogenesis of pyometra is not understood completely, it is clearly suggested that there are many predisposing factors like age, breeds, parity, neutering status, hormones and microorganisms contribute to the development of pyometra. Mostly it occurs during the diestrus phase of estrus cycle where intact corpus lutetium is present along with high concentration of progesterone. Therefore, progesterone-primed uterus can be highly vulnerable to the microbial infection by both the opportunistic as well as the pathogenic organisms. The repeated exposure of uterine endometrium to steroid hormones for an extended period of time may cause the development of a subclinical disease condition known as Cystic Endometrial Hyperplasia (CEH- an acute inflammatory condition of the uterine endometrium). The estrogen priming can up-regulate the receptors for both progesterone as well as estrogen on the endometrial surface. These hormones also cause relaxation of myometrium and closure of cervix. Initially CEH is manifested as hyper proliferation and hypersecretion of endometrial glandular cells which in turn leads to the development of cysts and accumulation of fluid in the uterine lumen. Since the uterine lumen is considered as sterile, CEH may leads to canine infertitlity. But this disease condition is a predisposing factor for the development of pyometra due to diminished innate immune function and increased adherence of microbes to endometrium. Hence, it is very important to diagnose pyometra during the initial stages and differentiate it from other uterine pathological conditions especially CEH. Till date, the most effective treatment suggested is the ovariohysterectomy yet other medical alternatives are also available. Thus, an early recognition, diagnosis, and appropriate intervention are of the utmost importance for the veterinarians in order to avoid fatal consequences [3-5].

Therefore, the article will be trying to highlight the recent findings on the histopathological, microbiological and immunological aspects for the diagnosis of pyometra.

2. The role of reproductive tract microbiota

The microbiota includes millions of bacteria which are normal inhabitants (symbiotic) and their presence ensures a healthy mucosal environment in the reproductive tract of a healthy animal. It is having an important role in maintaining the uterine microecological balance as well as in development of uterine diseases. The symbiotic microbe not only competes with pathogenic bacteria but also teach the host immune cells to produce cytokines/ chemokines to kill pathogenic microbes [6]. For example, during the estrus period, the relaxation of cervix allows the access of these microbial agents in to the uterine lumen but it will be immediately cleared by the phagocytic cells (primed by microbiota) of innate immune system. In CEH, due the inadequacy of proper defense mechanism, the microbial agents may get trapped in the uterine lumen and can survive in the cystic fluid which is an excellent medium for the growth of microbial agents. Further, proliferation of microbes causes endometritis and then eventually progressing to the accumulation of pus and inflammatory fluids in the uterus leads to the development of pyometra. Thus, interaction between the endometrium, endometrial microbiota and uterine immunity is crucial and abnormality of any one of these can lead to various uterine disease conditions like pyometra

Various reports suggest that *E. coli* is the foremost agent [7-11] yet other species also can cause disease. The virulence factors like endotoxins, lipopolysaccharides, membrane vesicles, peptidoglycan, and lipoteichoic acid can activate the inflammatory reaction [11]. During the multiplication of bacterial agent, it can disintegrate/or release these factors in to the surroundings and cause fever, lethargy, tachycardia, tachypnea, endotoxemia, septic shock disseminated intravascular coagulation, and generalized organ failure etc. [7, 12]. Thus, the virulence of the infectious agent, cytotoxic necrotizing factors and other inflammatory mediators are crucial in development of pyometra. Therefore, detection of these virulence factors (microbial biomarkers) for the identification of early microbial invasion of endometrium is critical for the clinical intervention in patients especially in animal with closed pyometra to prevent the septicemia and toxemia.

Most of the studies reports that the vaginal and uterine microbiota in healthy bitches is predominantly colonized by *Lactobacillus spp*. [13, 14] but other genus also reported. Rota *et al*., (2021) reported frequent isolation of different bacteria like *Acinetobacter spp*., coagulase-negative *Staphylococci* and *Bacillus spp.* from the different sites of pregnant uterus of healthy animal whereas *Pseudomonas aeruginosa*, *Micrococcus spp.*, *Moraxella spp*., *Macrococcus spp*., *Glutamicibacter spp., Stenotrophomonas spp. and Psychrobacter spp*. were only occasionally identified from these sites. Zheng *et al*., (2023) [16] suggest that the most prevalent microbes in the uterus of diseased dogs belonged to the genus *Pseudomonas*, *Escherichia-Shigella*, *Mycoplasma*, *Enterococcus*, *Haemophilus*, *Vibrio* and *Ralstonia*, *Mycoplasma*, *Enterococcus*, *Haemophilus* in the healthy animals. Another study suggests that the diet of the animal may influence the intestinal colonization of microbes like *E coli* which can be a risk factor for the development of pyometra [17]. It is already described that the microbial colonization which occurs in the earlier stages of life has a significant role in physiological and immunological development of an animal. For example, maintaining endometrial cell proliferation and apoptosis, preventing attachment of pathogenic microbes, helping attachment of microbial ligands and receptors to produce cytokines, chemokines, and antimicrobial substances to produce immunological response

Even after having enormous scientific evidences, the possibility of in utero microbiome and its role in fetus life is still on debate as many research finding suggest neonatal environment is sterile whereas many propose microbiome acquisition instead begins in utero [18-20]. Therefore, systematic evidence based scientific studies are required to find out the role of bacterial communities in the uterine environment and further development of pyometra.

3. The role of steroid hormones and histopathology

Pre-existing non-neoplastic reproductive disorders like cystic endometrial hyperplasia, peri-glandular fibrosis, lympho-plasmocytary endometritis, and adenomyosis have been observed in the uterus of apparently healthy animals. Among these subclinical pathologies, Cystic Endometrial Hyperplasia is found to be closely related to pyometra which is also known as Endometrial Hyperplasia-Pyometra Complex (EHP-C) [21]. Endogenous hormones (progesterone and estrogen) can jointly act as an inducer of uterine epithelial proliferation. Therefore, prolonged exposure to progesterone leads to proliferative condition during consecutive estrus cycle. It induces the growth and proliferation of endometrial glands, increases secretion, cervical closure, and cause suppression of myometrial contractions which is absolutely required during pregnancy [1]. But progesterone diminishes the mucosal defence mechanism by suppressing the activity of innate immune cells (Dendritic cells, neutrophils, Macrophages etc.) and favour the growth of microbes. Hormones and microbes are fundamental to development of EHP-C [22]. Therefore, the establishment of infectious microbes like *E coli* will be increased during prolonged progesterone level. When the host defense mechanism is diminished, these residential/ opportunistic pathogens take advantage and become more pathogenic. Though the influence of steroid hormone and microbial agents in the development of EHP-C is studied extensively, it is difficult to characterize the endometrial changes associated with every pathological stage of EHP-C.

Bacteria and its strains can produce various virulence factors which subsequently evoke inflammatory response causing significant changes in the uterine endometrium. Microbial infection can cause extravasation of the inflammatory cell due to chemokine and cytokine genes expression; antimicrobial peptides will be produced and there will be immediate action of innate immune system. Previous reports suggest that the inflammatory mediators like cyclooxygenase-2 inflammatory protein (COX-2) or prostaglandin-endoperoxide synthase 2, upregulation of metalloproteinase genes, vascular endothelial growth factor, were significantly elevated during pyometra [23]. Therefore, these studies suggest that pyometra is an inflammatory, proliferative, and vascular disorder.

4. Current diagnostic approach

The preliminary diagnosis of EHP-C is done by anamnesis, physical examination and laboratory testing including non-invasive diagnostic imaging like ultrasound/radiography. The clinical signs like anorexia, depression, polyuria, polydipsia, vomiting etc. are associated with this condition which may be confused with another systemic disease like renal failure,

hepatic disorders, diabetes etc. Also, signs of EHP-C primarily depend on either open or closed cervix pyometra. Open pyometra (OP) is less severe when compared to closed pyometra (CP) which is more severe and life threatening. During the early stages of pyometra, animal may not show any obvious outward clinical signs and diagnosis is mostly depends on imaging by ultrasound/radiography, laboratory testing of clinical samples, laparotomy/or laparoscopy etc.

4.1. Imaging by radiography and ultrasonography

4.1.1 Radiography

Radiographic imaging of the uterus may be used as a diagnostic tool for the detection of pyometra but should be backed up by other diagnostic procedures. Localized uterine enlargement may suggest a number of diseases including neoplasia, cystic endometrial hyperplasia, localized pyometra, hydrometra or mucometra, uterine stump granuloma or abscess, cystic uterine remnant and uterine adenomyosis [24]. In comparison with a lot of different diagnoses attributed to radiographic findings, ultrasonographic imaging of the uterus presents a much more specific technique [25].

4.1.2 Ultrasonography

Since last few decades, the diagnostic ultra-sonography (DUS) gained increased acceptance for the evaluation of reproductive disorders like fibrosis, cysts, endometrial disorders like pyometra mucometra etc. It is considered as superior to radiographic imaging as it can be used for both the real time qualitative and quantitative assessment of pathological condition and also doesn't involve any hazardess ionizing radiation. The integrity of endometrium, thickness of uterine wall, uterine enlargement and cystic endometrial glands can be evaluated with DUS for diagnosis of CEHP-C. The differentiation of fluid filled conditions of uterus like pyometra and mucometra can be done by examining uterine blood flow. Evaluations of biometric parameters like internal and external uterine diameter, thickness of uterine horn wall behind the uterine bifurcation are important for the selection of medical intervention and prognosis [26]. Advance DUS like Doppler ultrasonography, elastography, three-dimensional (3D) and four-dimensional (4D) ultrasound, and contrast-enhanced ultrasound (CEUS) are available for diagnosis of pyometra [27, 28].

4.2 Clinical pathology

4.2.1. *Biochemical and physiological analysis*

The differential diagnosis of pyometra from other uterine pathological conditions can be done by examining the uterine cytology. Clinical samples like vaginal discharge may be used for cytological examination for differentiating open-cervix pyometra from mucometra [7]. Degenerative neutrophils are frequently seen and extra/intra cellular microbes like bacteria may be observed on cytological examination of open-cervix pyometra. In closed-cervix pyometra, a marked peripheral leucocytosis is commonly observed especially Leucocytosis with neutrophilia and lymphocytosis [29-32]. When a differential cell count is performed a left shift is also a common finding [33]. A packed cell volume of 21-48% was reported by Stone *et al.*, 1988 in animals with normocytic, normochromic anaemia. The anaemia may be due to the bone marrow suppression by the toxins liberated by the pathogens, decreased erythrocyte viability and could also be due to the diapedesis of the R.B.C s into the lumen of the uterus and shortened life span of circulating erythrocytes and associated iron deficiency [29, 35].

The biochemical analysis of serum shows abnormalities like hypo-albuminemia azotemia, hyper-gammaglobulinemia etc. [30]. Other serum chemistries like ALT, AST, ALP, serum urea nitrogen, creatinine which are common in other disease condition also found to be elevated in pyometra [36, 37]. Marked tissue damage of uterus during pyometra especially due to microbial infection can be the reason for the elevated serum urea nitrogen, creatinine levels and neutrophilic values [34, 38, 39]. Hypo-albuminaemia and hyper-globulinaemia could be due to acute phase reaction and dehydration and/ or chronic antigen stimulation of the immune system respectively [40]. The circulating microbes (from Uterus) and its toxins may cause hepato-cellular damage and intra-hepatic cholestasis leading to rise of ALT, AST and ALP [31]. Various reports suggest that C-reactive proteins (CRP) are elevated significantly in pyometra but as it is non-specific it may be used for the diagnosis. Dabrowski *et al.*, 2007 concluded in their study that acute phase proteins like C-reactive protein (CRP), serum amyloid a component (SAA) and haptoglobin (Hp), can be used for canine pyometra as routine diagnostic markers of early post-operative complications.

In complicated cases of pyometra, other internal organs may be affected like liver, kidney, lungs etc. Therefore, other organ specific indicators may also suggestive of uterine abnormalities. Renal abnormalities caused by bacterial endotoxins like insufficient renal tubules to concentrate urine which intern affect the specific gravity of urine further leading to the clinical signs of polyuria and polydipsia. But these findings are inconsistent as dehydration may also affect urine specific gravity [42].

4.2.2 Histopathological analysis

It is based on various criteria for identification of chronic purulent metritis, purulent endometritis or purulent metritis [43]. Macroscopically distended uterus with irregular endometrial surface was observed in chronic purulent metritis. Similarly, microscopic pictures show irregular numbers of neutrophils, plasma cells and lymphocytes in the mucosa which are also present in the myometrium to a lesser extent. The hyperplastic-cystic endometrial epitheliums along with columnar cells are suggestive of progesterone influence on the endometrium. These cysts may contain large amounts of neutrophils. Fibrosis and/or erosions in the mucosa may be seen.

In purulent endometritis, observed inflammatory cells were mostly neutrophils and cellular infiltration was limited to the endometrium, whereas in purulent metritis inflammatory changes were also present in deeper layers of the uterine wall.

Gross and histo–pathological examination of pyometric ovary and uterus was extensively done by Ravishankar *et al.* (2004) in table 2 [38].

Table 1: Show Organs, Gross pathology and Histopathology

Organs	Gross pathology	Histopathology
Ovaries	**Multiple corpus lutea**	**Multiple corpus lutea**
Uterus	**Horns:** Dilated and distended and sacculation along the length. **Pus:** Creamy to chocolate-coloured in some cases and viscid in majority. **Wall:** Thinned out in closed pyometra and thick in open cases. **Endometrium:** Irregularly thickened, ecchymotic ulcerations, bran like necrotic sherds.	**Endometrium:** Cytoplasmic vacuolations roughly spherical vesicular nuclei often pushed to the periphery, focal necrosis and hyperplasia. Neutrophilic and plasma cell infiltration. **Glands:** Multiple cystic distentions containing eosinophilic glandular exudates infiltrated with neutrophils and mononuclear cells in and around the glands. **Myometrium:** Extension of inflammation. Neutrophilic infiltration, haemorrhages and plasma cell infiltration around arteries.

4.3. Microbiological and immunological analysis

The early diagnosis is crucial for appropriate clinical management which intern affect outcome of the diseases. Factors like microbes, surgical trauma, tumors can increase the inflammatory response by releasing inflammatory mediators which may affect the body systemically. Acute phase proteins, cytokines or tryptophan metabolites are some of the inflammatory mediators produced in response to bacteremia, septicemia and endotoxemia which can further aggravate inflammation and leads to shock and death. Therefore, inflammatory mediators can be considered as early indicator of endometritis. Also, the composition of endometrial microbiome and inflammatory response during various endometrial pathological condition varies [6] which require more studies to identify the immunological markers for the prevention and treatment of pyometra [23, 44, 45].

The traditional laboratory techniques like isolation, staining, biochemical test etc. are used for primary identification of microbial agents, (mostly bacteria) which is responsible for pyometra. However, isolation and identification may consider as gold standard but have limitation in case of non-cultivable microbes. Therefore, many times, non-cultivable potential pathogens were not identified / reported. In such case, molecular markers may be identified using high throughput next generation and metagenomic sequencing. A 16S rRNA gene based identification of microbiome revealed that relative abundance of the genus belonging to the family *Pasteurellaceae*, *Porphyromonadaceae* and *Fusobacteriaceae* instead *Enterobacteriaceae* [45]. Many studies reported the presence of microbes in utero by detecting bacterial specific DNA from samples collected from amniotic fluids, meconium, placental attachment point etc. However, molecular diagnosis must be done with great care as these data have remained as contentious due to underlying contamination issues

4.4 Conclusion

Recent advancements in uterine microbial-ecology, serology and endometrial transcript-based biomarkers have made it feasible to diagnose pyometra at early stages in dogs and differentiate it from cystic endometrial hyperplasia. Since CEH and pyometra have distinct clinical, histopathological and immunological characteristics, they should be classified separately. These developments in pyometra research are encouraging and holds potential for improving the diagnosis and management of this condition in dogs.

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