UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

# *Escherichia coli* - host interactions in the pathogenesis of canine pyometra

**Sofia Correia Rosa de Barros Henriques**

Orientador(es): Doutora Luísa Freire Leal Mateus
Doutora Maria Elisabete Tomé Sousa Silva

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Ciências Biológicas e Biomédicas

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"Science is the great antidote to the poison of enthusiasm and superstition."

Adam Smith (in *Wealth of Nations*, 1776)

"It is not the strongest of the species that survives, not the most intelligent that survives. It is the one that is the most adaptable to change."

Charles Darwin (in *Origin of Species*, 1859)

*To my grandparents Mariette and Nuno, Lai and Luís to my parents Helena e Carlos and to my dear sister Ana for all the unconditional love and support*

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**Thesis title – *Escherichia coli* - host interactions in the pathogenesis of canine pyometra**


## Abstract

Canine pyometra develops as a result of a complex interaction of etiological and physiopathological factors, such as the virulence and type of the bacteria and the individual host defence mechanisms. Since *Escherichia coli* is the most common bacterium isolated from uterus of bitches with pyometra, one main objective of this work was to characterize *E. coli* virulence potential, and to evaluate the role of its virulence factors (VF) and traits in the pathogenesis of canine pyometra (Chapters IV, V and VI). A second main objective was to evaluate the innate immune mechanisms within the uterus and their role in *E. coli* recognition (Chapters III and VI).

Results indicate that: i) although no single VF genes or virulence traits were associated with *E. coli* pyometra isolates, these isolates were mainly from the highly virulent phylogenetic group B2, which are characterized by a high number of uropathogenic *E. coli* VF genes and pathogenicity-associated islands markers; ii) Toll-like receptors were involved in the activation of the inflammatory response associated with pyometra; iii) β-hemolytic *E. coli* infection was associated with the occurrence of metritis and with an higher uterine tissue damage; iv) α-hemolysin (HlyA) contributes to the virulence of β-hemolytic *E. coli*, by inducing endometrial epithelial and stromal damage and a compromised early uterine immune response.

Overall, these findings provide new relevant insights into the role of the pathogen-specific modulation of host immunity, which may influence the severity of disease and its clinical outcomes. Also, HlyA is a promising target for a vaccine, with the objective to induce an immunity that can block the binding and action of this toxin.



**Keywords**: *Escherichia coli*, virulence traits, α-hemolysin, innate immunity, canine pyometra.

**Título da dissertação – Interacão hospedeiro-*Escherichia coli* na patogenia da piómetra na cadela**

## Resumo

A piómetra é uma doença comum do trato genital de cadelas adultas, durante a fase de diestro. A piómetra desenvolve-se como resultado de uma complexa interação de fatores etiológicos e fisiopatológicos. Entre estes, incluem-se a influência hormonal no útero, alterações estruturais no endométrio - como a hiperplasia quística (HQE) -, o tipo de bactérias e o seu potencial de virulência, e os mecanismos de defesa do hospedeiro. O trabalho desenvolvido nesta tese baseou-se no estudo do potencial de virulência de *Escherichia coli* (*E. coli*) (Capítulos IV e V) e nos mecanismos de imunidade inata do útero (Capítulos III e VI).

Tendo em conta a elevada prevalência de *E. coli* nos casos de piómetra (82-100% dos casos) e nas infeções do trato urinário (54 – 68%) e o facto de aquelas estirpes serem provenientes da flora fecal do animal e não de um clone específico disseminado entre animais, procedeu-se à comparação do potencial de virulência de *E. coli*, isolada de piómetra, de cistites e de fezes de cadela (Capítulo IV). Os resultados indicam que as estirpes de *E. coli*, que colonizam o útero, têm um elevado potencial de virulência, possuindo um grande número de genes que codificam para fatores de virulência (FV) e ilhas de patogenicidade (PAIs). No entanto, existem estirpes de *E. coli* isoladas de cistite e de origem fecal com as mesmas características, o que sugere que poderão induzir piómetra, em cadelas suscetíveis. De particular importância, foi a observação de que cerca de 50% das estirpes de *E. coli* isoladas de piómetra eram β-hemolíticas. A prevalência dos isolados pertencentes ao grupo filogenético B2 foi maior nos casos de piómetra (94%) do que nos casos de cistite (48%) ou do que nos de origem fecal (39%). No entanto, independentemente da origem dos isolados, o número médio de PAIs e de genes que codificam para FV foi maior nos isolados pertencentes ao grupo filogenético B2, comparativamente aos outros grupos filogenéticos. Verificou-se também que o reto poderá funcionar como um reservatório de estirpes potencialmente patogénicas dos grupos filogenéticos B2 e D. Esta observação tem especial importância pois sabe-se que as estirpes de *E. coli* uropatogénicas isoladas de cães e humanos são similares em relação ao seu

serotipo, tipo clonal, grupo filogenético e perfil de virulência. Isto sugere que os cães podem servir como reservatórios de bactérias potencialmente virulentas que podem ser transmitidas ao homem.

Na primeira semana pós-parto*, E. coli* é a bactéria mais frequentemente isolada do conteúdo uterino de vacas de leite que desenvolvem infeções uterinas puerperais. No entanto, a associação, entre o perfil de virulência de *E. coli* e o desenvolvimento de metrite puerperal ou clinica, é controverso e, em muitos dos casos, a infeção resolve-se espontaneamente. Na cadela, as piómetras por *E. coli* estão associadas, em 50% dos casos, à síndrome de resposta inflamatória sistémica, a qual é potencialmente letal na ausência de terapêutica adequada. Numa tentativa de relacionar o potencial de virulência de *E. coli* com as diferentes evoluções da metrite clinica na vaca e da piómetra na cadela, compararam-se características genómicas dos isolados de *E. coli* (Capítulo V). Os resultados mostram que as estirpes de *E. coli* isoladas de vacas com metrite clinica pertencem maioritariamente aos grupos filogenéticos B1 e A, são geneticamente distintas das estirpes de piómetra e apresentam um menor número de genes que codificam para fatores de virulência, sendo por isso consideradas estirpes de menor potencial de virulência.

A resposta uterina à infeção é composta por mecanismos da imunidade inata e adaptativa. A resposta inata é desencadeada pelo reconhecimento de padrões moleculares associados aos agentes patogénicos, por recetores do tipo Toll (TLRs), induzindo uma reacção inflamatória. Os resultados apresentados no Capítulo III permitem concluir que o útero da cadela tem capacidade de reconhecer uma grande variedade de ligandos - através da activação dos TLRs - e desenvolver uma resposta inflamatória contra vários tipos de microorganismos. Verificou-se, também, que a transcrição e expressão dos TLRs 2 e 4 encontram-se significativamente diminuídas no início de diestro, o que pode contribuir para a maior susceptibilidade do útero à infeção por *E. coli,* nesta fase. Os resultados apresentados no Capítulo VI demonstram que, nos casos de piómetra a resposta inflamatória, mediada pelos TLRs, foi caracterizada por uma reação inflamatória exuberante, demonstrada pelo influxo de células de reação inflamatória no útero e por um aumento na transcrição de genes que codificam para citocinas pró-inflamatórias (IL-1β, IL-6, IL-8) e anti-inflamatórias (IL-10 e TGFβ). Observação relevante foi que, nos casos de piómetra por *E. coli* β-hemolítica, há um aumento significativo da

ocorrência de metrite e uma maior destruição do endométrio, bem como um aumento significativo da transcrição dos genes que codificam para as citocinas IL-1β e IL-8, quando feita a comparação com as piómetras por *E. coli* não hemolítica.

Tendo em conta os resultados apresentados anteriormente, bem como a elevada percentagem de isolados de *E. coli* β-hemolítica nos casos de piómetra, foi avaliado o papel da α-hemolisina (HylA) na patogenia da piómetra. Para este fim, recorreu-se à estimulação *in vitro* de culturas primárias de células epiteliais e do estroma do endométrio canino com uma estirpe não hemolítica (Pyo14), uma estirpe β-hemolítica (Pyo18) e um mutante isogénico da HylA (Pyo18*ΔhlyA*, perda de função). Os resultados obtidos mostram que o efeito citopático da HylA é maior nas células do estroma do que nas células do epitélio do endométrio. Este facto poderá estar associado à maior destruição do endométrio e ao maior número de casos de metrite por nós observado. Foi também demonstrado que estas estirpes de *E. coli* induzem respostas imunitárias diferentes nos dois tipos celulares. Nas células do estroma, a HylA esteve associada a uma diminuição da transcrição dos genes que codificam para as citocinas IL-1β, TNFα e IL-10. Pelo contrário, a estimulação celular com o mutante isogénico levou a um aumento da transcrição destas citocinas. A ação da HylA na inibição da transcrição destes mediadores da inflamação poderá permitir a multiplicação e invasão do endométrio por *E. coli* β-hemolítica, antes que se estabeleça uma reação inflamatória adequada.

Em súmula, os trabalhos que compõem esta tese permitem concluir que: i) as estirpes de *E. coli* isoladas de piómetra são principalmente do grupo filogenético B2, sendo caracterizadas por uma maior número de FV e PAIs; ii) os recetores do tipo Toll estão envolvidos na ativação da resposta inflamatória nos casos de piómetra; iii) a infecção por *E. coli* β-hemolítica está associada a metrite e a uma maior destruição do tecido uterino; iv) a α-hemolisina contribui para a virulência de *E. coli* β-hemolítica ao induzir uma destruição das células epiteliais e do estroma do endométrio e comprometer precocemente a resposta imunitária uterina.

Estes resultados aportam conhecimento novo sobre o papel de *E. coli* na modulação da resposta imunitária uterina e na patogenia da piómetra. Pelo seu papel na expressão da virulência de *E. coli*, a HlyA é uma potencial candidata para o desenvolvimento de uma

vacina. Assim, os TLRs poderão ser considerados promissores alvos terapêuticos, dado o seu envolvimento na resposta inflamatória associada à piómetra.

**Palavras-chave:** *Escherichia coli*, perfil de virulência, α-hemolysina, imunidade inata, piómetra de cadela

**TABLE OF CONTENTS**

**LIST OF FIGURES**

**LIST OF TABLES**

## LIST OF ABBREVIATIONS AND SYMBOLS

% - percentage

µg - microgram

µL - microliter

a - anestrus

ABC - ATP-binding cassete

afa - afimbrial adhesins

AP-1 - activator protein 1

APEC - avian pathogenic *E. coli*

APC - antigen presenting cells

astA - heat-stable cytotoxin associated with enteroaggregative *E. coli* toxin

ATP - Adenosine triphosphate

Bp - base pairs

BSA - bovine serum albumin

cDNA - complementary DNA

cdt - cytolethal distending toxin

CEH - cystic endometrial hyperplasia

CFU - colony-forming unit

CHEF - clamped homogenous electric field

chuA - *E. coli* heme transport protein

CLSI - Clinical and Laboratory Standards Institute

cnf1 - cytotoxic necrotizing factor 1

CO2 - carbon dioxide

csg - curli fibres

cvaC - microcin CoIV

DAB - substrate diaminobenzidine

DAEC - diffusely adherent *E. coli*

DAF - decay-accelerating factor

DCs - dendritic cells

DMEM - Dulbecco's Modified Eagle Medium

DNA - deoxyribonucleic acid

dra - dr fimbriae

dsRNA - double stranded RNA

eaeA - intimin

EAEC - enteroaggregative *E. coli*

*E. coli* - *Escherichia coli*

ED - early diestrus

EHEC - enterohamerrhagic *E. coli*

EIEC - enteroinvasive *E. coli*

EnPEC - endometrial pathogenic *E. coli*

ent - enterobactin

EPEC - enteropathogenic *E. coli*

ETEC - enterotoxigenic *E. coli*

ExPEC - extra-intestinal *E. coli*

fecA - ferric citrate

fepA - iron-enterobactin outer membrane transporter

fhuA - yersiniabactin system

fhuE - coprogen

fhuF - ferrioxamine B

fim - type 1 fimbriae

foc - F1C fimbriae

fyuA - yersiniabactin system

g - grams

h – hour

H - hemolytic

HBSS - Hanks' Balanced Salt Solution

hlyA - alpha-hemolysin

hlyE - hemolysin E

Hma - heme acquisition protein

HPI - high-pathogenicity island

HYP - hypervariable domain

ibeA - invasion of brain endothelium

IFN - interferon

IgG - immunoglobulin G

IHC – immunohistochemistry

IKK - inhibitor of κ B kinase

IL - interleukin

IM - inner membrane

iNOS - inducible nitric oxide synthase

IPEC - enteric/diarrheagenic *E. coli*

IRAK - IL-1 receptor-associated kinase

IRF - IFN regulatory factor

iroN – catecholate siderophore receptor

ITS - Insulin-Transferrin-Selenium

iucD – aerobactin system

iutA – aerobactin

kpsMT - capsular polysaccharide synthesis

LBP - LPS binding protein

LH - luteinizing hormone

LPS – Lipopolysaccharide

LRR - leucine-rich repeats

HSP - heat shock proteins

LH - luteinizing hormone

LTA - lipoteichoic acid

MAP - mitogen-activated protein

M - mucoide

MAPK – mitogen-activated protein kinase

mCD14 - membrane-bound CD14

MD/LD - mid/late diestrus

MD-2 - myeloid differentiation factor-2

mg - milligrams

MHC - major histocompatibility complex

MIC - minimum inhibitory concentration

min – minutes

ml - milliliter

MPEC - mammary pathogenic *E. coli*

mM – milimol

MRHA - mannose-resistant hemagglutination

mRNA – messenger RNA

MSHA - mannose-sensitive hemagglutination

MyD88 - myeloid differentiation primary response 88

N - number

NAPs - natural antimicrobial peptides

NF-κB - nuclear factor kappa B

NH - non-hemolytic

NMEC - neonatal meningitic *E. coli*

◦C – grad Celsius

OAZ1 - ornithine decarboxylase antizyme 1

OE - oestrus

OHE – ovariohysterectomy

OM - outer membrane

OTUs - operational taxonomic units

OVX – ovariohysterectomy

P4 - progesterone

PAIs - pathogenicity-associated islands

PAMPs - pathogen associated molecular patterns

pap - P fimbriae adhesin

PBP - periplasmic binding protein

PBS - Phosphate-buffered saline

PCR - polymerase chain reaction

PE - proestrus

PFGE - pulsed-field gel electrophoresis

Pg - picogram

PG – prostaglandins

PGE2 - prostaglandin E2

PGES - prostaglandins E2 synthases

$PGF_{2\alpha}$ - prostaglandin $F_{2\alpha}$ metabolite

PGFM - prostaglandin $F_{2\alpha}$ metabolite

PGFS - prostaglandins F2α synthases

pH - potential of Hydrogen

PMN - polymorphonuclear leukocyte

pmol – picomol

PRRs - specialized pattern recognition receptors

PTGS2 - prostaglandin-endoperoxide synthase 2

qRT-PCR - quantitative reverse transcription polymerase chain reaction

R - rough

RLHs - rig-like helicases receptor

RNA - ribonucleic acid

RT-PCR - reverse transcription polymerase chain reaction

RTX - repeats-in-toxin

S - smooth

SEPEC - septicaemia associated *E. coli*

SIRS - systemic inflammatory response syndrome

sitA - periplasm iron binding protein

sfa - S fimbriae

spp - species

ssRNA - single stranded RNA

stx - shiga toxin

TcpC - Toll/interleukin-1 receptor (TIR) domain-containing protein

TGF - Transforming growth factor

TIR - translocated intimin receptor

TIRAP/MAL - TIR-containing adaptor protein

TLRs - Toll-like receptors

TLR2 - Toll-like receptor 2

TLR4 - Toll-like receptor 4

TNF - tumor necrosis factor

TNFR - tumor necrosis factor receptor

TRAF - TNFR-associated factor

TRIF - TIR-domain-containing adapter-inducing interferon-β

TRAF6 - TNF receptor-associated factor 6

TRAM - TIR-domain-containing adaptor molecule 2

tRNA - Transfer ribonucleic acid

traT - serum survival factor

U - units

u/ml - units/milliliter

UPEC - uropathogenic *E. coli*

UPGMA - unweighted average linkage

usp - uropathogenic specific protein

UTI - urinary tract infection

VF - virulence factor

VFGs - virulence factor genes

# CHAPTER I – GENERAL INTRODUCTION AND OBJECTIVES

Pyometra is a common and serious uterine disorder that develops in 25% of all intact female dogs, causing a variety of clinical and pathological signs. *Escherichia coli* (*E. coli*) is the most common bacterium isolated from the uterus of bitches with pyometra (82-100%). Its presence is normally associated with severe systemic signs. If left untreated is lethal and patients may develop endotoxemia, sepsis or septic shock. Apart its relevance for the canine species, studies on pyometra disease mechanisms may provide important insights into the mechanisms operating in human bacterial infection and sepsis. Despite several studies on the etiology of this disease, the pathogenesis of pyometra is still not completely understood. In particular, little is known regarding the host-pathogen interactions occurring in the pathogenesis of pyometra. The current knowledge on the pathogenicity of *E.coli* isolates from canine pyometra cases is based on the genomic identification of classical virulence factor genes (VFGs) (Chen *et al*., 2003; Siqueira *et al*., 2009). Although pyometra *E. coli* share virulence factor (VF) of uropathogenic *E. coli* (UPEC) strains, key VF essential for *E. coli* pathogenicity in pyometra cases are still unknown. Similarly, the role of *E coli* in the pathogenesis of the puerperal uterine infection of the cow is also unclear. It has been implicated in early ovarian disturbances and appears to increase the susceptibility of the uterus to subsequent infections with *Trueperella pyogenes* and Gram-negative anaerobes, the former being relevant to the establishment and persistence of uterine infection (Mateus et al., 2002; Sheldon et al., 2009). However the outcomes of these two conditions are very different and may be linked to different *E. coli* virulence traits, among other reasons.

The endometrium is the first line in defending against invading microbial pathogens. It recognizes pathogen-associated molecular patterns (PAMPs) shared by pathogens via innate immune receptors. Toll-like receptors (TLRs) are a large class of ancient innate immunity receptors which can recognize conserved components of pathogens or PAMPs and initiate innate immune response (Horne *et al*., 2008). An enhanced understanding of innate immune mechanisms within the female reproductive tract and their role in bacterial recognition may provide insights into the pathogenesis of uterine diseases and associated deleterious sequelae. The understanding of the underpinning mechanisms of pyometra and the potential role by which *E. coli* subvert host innate immunity, disrupting uterine function, is crucial to develop surrogate measures to alleviate the negative impacts of uterine infection.

Therefore, the research work presented in this thesis aimed to improve our understanding of the host-pathogen interactions in canine pyometra, and more specifically:

1. To evaluate if changes in toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4) transcription and expression during the oestrous cycle may be associated with predisposition to the development of pyometra in the bitch.

2. To characterize the virulence potential of pyometra, UTI and feces *E. coli* in order to explain their ability to colonize and infect either the uterus or the urinary tract.

3. To compare the molecular and phenotypic characteristics of *E. coli* isolates recovered from the uterus of cows with clinical metritis and of bitches with pyometra, in an attempt to correlate their virulence potential with the different clinical outcomes of the diseases.

4. To evaluate the inflammatory response of canine endometrial cells to uteropathogenic *E. coli*, focusing on the role of α- hemolysin in epithelial and stromal cells.

The above studies were converted into four articles, submitted for publication in international refereed and indexed journals, and constitute the four chapters of the experimental work included in this thesis as follow:

1. **Oestrous cycle-related changes in production of toll-like receptors and prostaglandins in the canine endometrium.**
   Silva, E.[1], Henriques, S.[1], Brito, S., Ferreira-Dias, G., Lopes-da-Costa, L., Mateus, L. (2012). *Journal of Reproductive Immunology*, 96 (1-2): 45-57. doi:10.1016/j.jri.2012.07.003

[1]These authors contributed equally to this work

2. **Virulence genotypes of *Escherichia coli* canine isolates from pyometra, cystitis and fecal origin.**

   Mateus, L., Henriques, S., Merino, C., Pomba, C., Lopes-da-Costa, L., Silva, E. (2013). *Veterinary Microbiology*, 166 (3-4): 590-594. doi:10.1016/j.vetmic.2013.07.018

3. **Genotypic and phenotypic comparison of *Escherichia coli* from uterine infections with different outcomes: Clinical metritis in the cow and pyometra in the bitch.**

   Henriques, S., Silva, E., Lemsaddek, A. Lopes-da-Costa, L., Mateus, L. (2014). *Veterinary Microbiology*, 170 (1-2): 109-116. doi:10.1016/j.vetmic.2014.01.021

4. **Immunomodulation in the canine endometrium by uteropathogenic *Escherichia coli***

   Henriques, S.[1], Silva, E.[1], Silva, M.F., Carvalho, S., Diniz, P., Lopes-da-Costa, L., Mateus, L. (2016). Submitted to *Veterinary Research*

   [1]These authors contributed equally to this work

# CHAPTER II - LITERATURE REVIEW

## 1. Canine Pyometra

Canine pyometra is a chronic disease characterized by the accumulation of pus in the uterine lumen of sexually mature intact bitches. The disease is associated with a variety of clinical and systemic signs, and occurs during a phase of progesterone dominance, usually diagnosed from 4 weeks to 4 months after estrus (Fransson & Ragle, 2003; Pretzer, 2008; Smith, 2006). Around 25% of all intact female dogs develop pyometra by 10 years of age (Dow, 1958; Egenvall et al., 2001; Niskanen & Thrusfield, 1998). Reported age range at time of development of pyometra is 8 months to 15 years with a mean age of 8.6-9.9 years (Bigliardi et al., 2004; Niskanen & Thrusfield, 1998).

Pyometra develops as a result of a complex interaction of etiological factors. These factors include changes within the endometrium, the hormonal influence on uterine environment, the virulence and type of the bacteria and the individual defense mechanisms (Mateus & Eilts, 2010).

Cystic endometrial hyperplasia (CEH) develops after repeated estrous cycles in the bitch and predisposes uterus to a secondary infection that leads to pyometra (Fransson, 2003). In this way, for several years the term CEH/pyometra complex was used when referring to these two entities. However pyometra can also occur without CEH, and clinical and histopathologic findings suggest that CEH and pyometra should be classified separately into two entities. Both CEH and pyometra have similarities with each other, except for the inflammatory reaction observed in pyometra (De Bosschere, Ducatelle, Vermeirsch, Van Den Broeck, & Coryn, 2001).

Clinical signs in bitches with pyometra vary depending on cervical patency (open or closed pyometra) and with the type of bacteria. Vulvar discharge is often evident if the cervix is open (reported in 58 to 98 % of bitches), and the discharge's characteristics may vary depending on the bacteria: muco-purulent, purulent (associated more often with *Streptococcus* spp) or sanguinopurulent (similar to tomato soup; associated more often with mucoid or haemolytic *E. coli*), white to red-brown in color and foul smelling (Gilbert, Nothling & Oettlé, 1989; Hagman et al., 2006a). A bitch with a closed cervix pyometra more commonly has abdominal distension. The onset of clinical signs may be acute or gradual, and in general, are more severe with a closed cervix pyometra (Smith, 2006). Common clinical signs associated with the pyometra are lethargy and anorexia,

polydipsia and polyuria, abdominal pain, vomiting or diarrhea and dehydration (Bigliardi et al., 2004; England, Freeman, & Russo, 2007; Gilbert et al., 1989; Hagman et al., 2006a).

Although less frequent, pyometra also can appeared associated with the systemic inflammatory response syndrome (SIRS) (Fransson, Lagerstedt, Hellmen, & Jonsson, 1997; Purvis & Kirby, 1994). Uncontrolled production of inflammatory mediators, such as tumor necrosis factor (TNF), interleukin-1 (IL-1) and interleukin-6 (IL-6), and platelet activating factor may provoke irreversible damage to internal organs or septic shock, which in some cases may lead to death (Manfra, Matthiesen & Nichols, 1989; Purvis & Kirby, 1994).

Bitches having higher plasma concentrations of endotoxins are associated with poorer prognosis for survival (Okano, Tagawa, & Takase, 1998). Endotoxins strongly stimulate prostaglandin synthesis, with prostaglandin $E_2$ ($PGE_2$) further contributing to the suppressed activity of cellular immunity during diestrus (Silva et al., 2010). Measuring blood concentration of the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) metabolite (PGFM) also provides a good indicator of endotoxin release in bitches with pyometra, and helps in the differentiation between pyometra and CEH (Hagman et al., 2006a). The increased systemic PGFM observed with pyometra probably originates from the endometrial synthesis of prostaglandins, which results from the stimulation of the uterus by bacteria (Silva et al., 2010). The safest treatment of pyometra is ovariohysterectomy (OHE), normally performed a soon as the general condition of the dog is stable. Medical treatment is an option in some selected cases to preserve fertility (Verstegen et al., 2008) . All bitches with pyometra, whether treated medically or with OHE, should be treated with systemic antibiotics.

### 1.1. Hormonal Component

Over the years, the association between pyometra and diestrus has been well established however the exact mechanism that leads to pyometra development is still unknown (Mateus & Eilts, 2010).

Canine pyometra disease occurs during diestrus stage when uterus is under progesterone influence. Bacteria may gain access into the uterus during pro-estrous and estrous phases

(J. R. Watts, Wright, & Lee, 1998). It is suggested that low-grade uterine infection is partially responsible for some of the endometrial proliferation that occurs early in the pathogenesis of CEH-complex (Arora, Sandford, Browning, Sandy, & Wright, 2006; Noakes, Dhaliwal & England, 2001; Nomura, 1983; Schlafer & Gifford, 2008).

The progesterone-sensitized uterus is suitable, not only for pregnancy, but also for bacterial infection, since progesterone stimulates endometrial glandular secretions, decreases myometrial contractions¸ induces functional cervical closure (Nelson & Feldman, 1986; Fransson & Ragle, 2003; Mateus & Eilts, 2010) and depress the local immune response by creating an anti-inflammatory environment (Sugiura et al., 2004; Tibbetts, Conneely, & O'Malley, 1999). Also, progesterone causes an increased binding of *E. coli* to the endometrium (Ishiguro et al., 2007; Leitner, Aurich, Galabova, Aurich, & Walter, 2003). Recent research involving the induction of canine pyometra by inoculating *E. coli* into the uterus demonstrated that, on days 11-20 and 21-30 after the luteinizing hormone (LH) peak, the uterus was most susceptible to infection (Tsumagari et al., 2005). In contrast, estrogens induce proliferation of endometrial glands, reduce the susceptibility of the endometrial epithelium to *E. coli* adhesion (Nishikawa & Baba, 1985) and induce a pro-inflammatory response within the uterus (Tibbetts, Conneely, & O'Malley, 1999) by increasing the production of bactericidal lactofferrin, the major estrogen-inducible protein in the uterus, that kills bacteria and modulates inflammatory and immune response (Teng et al., 2002) . Although estrogen seems to play a less important role, it appeared to enhance the endometrial response to progesterone (Teunissen, 1952; Nelson & Feldman, 1986).

At moment, there is no conclusive evidence that pyometra is caused by a disturbance in either hormone production or in the uterine response to progesterone and estrogen (De Bosschere, Ducatelle, Vermeirsch, Simoens & Coryn, 2002; Dhaliwal, England & Noakes, 2002). Peripheral blood concentrations of estrogen and progesterone in bitches with CEH/pyometra complex are not abnormally elevated compared to normal bitches (Chen, Wright & Lee, 2001). This has led to speculation that hormone receptors may thus play a role in the pathogenesis of CEH/pyometra (De Cock, Vermeirsch, Ducatelle & De Schepper, 1997; Dhaliwal, England & Noakes, 1999; De Bosschere et al., 2002; Nomura, Kawasoe & Shimada, 1990). Estrogen and progesterone receptor expression has been shown to be increased in the uteri of bitches with CEH, but not in the uteri of bitches with

pyometra, when compared with healthy bitches (De Bosschere et al., 2002; De Cock et al., 1997; Ververidis et al., 2004). In contrast, an overall reduction in both receptors in CEH/pyometra cases has been described (Dhaliwal et al. 1999).

### 1.2. Bacteriologic component

The bacteria most commonly isolated from the uterus in cases of pyometra of bitches are *E. coli* (around 90% of the cases). Its presence is normally associated with highly severe systemic signs and a potentially life-threatening situation. However, other bacteria that may be isolated include *Streptococcus* spp, *Klebsiella* spp, *Staphylococcus aureus*, *Pasteurella* spp, *Proteus* spp and *Pseudomonas* spp. (Chen, Wright, Lee, & Browning, 2003; Dhaliwal, Wray, & Noakes, 1998; Fransson et al., 1997; Hagman & Kühn, 2002). These organisms are also those that are most commonly isolated from the vagina of normal bitches (Watts, Wright, & Whithear, 1996) and can ascend to the uterus during pro-estrus and estrus (Kustritz, 2006). *E. coli* associated with canine pyometra originates from the normal intestinal flora of bitches and is not derived from any specific clonal type that is epidemically spread between animals (Chen et al., 2003; Hagman & Kühn, 2002; Wadås, Kühn, Lagerstedt, & Jonsson, 1996). *E. coli* associated with pyometra are characterized by the presence of several virulence genes normally found in UPEC strains (Chen et al., 2003; Siqueira et al., 2009). 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 (Hagman & Kühn, 2002; Wadås et al., 1996).

## 2. *Escherichia coli*

*E. coli* strains can be commensal as part of normal microbial flora or can cause various infection diseases in immunosuppressed host like intestinal infections (enteric/diarrheagenic *E. coli*, IPEC) or extra-intestinal infections (extra-intestinal *E. coli*, ExPEC) (Bélanger et al., 2011; reviewed by Bien, Sokolova, & Bozko, 2012; Picard et al., 1999). Intestinal infections can be caused by different *E. coli* pathotypes such as enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohamerrhagic (EHEC), enteroaggregative (EAEC), enteroinvasive (EIEC) and diffusely adherent (DAEC) *E. coli*

(Bélanger et al., 2011; Constantinou, Young, Clements, & Frankel, 2012; reviewed by Köhler & Dobrindt, 2011). The ExPEC includes UPEC *E. coli*, neonatal meningitic (NMEC), septicaemia associated (SEPEC) and avian pathogenic *E. coli* (APEC) (reviwed by Antão, Wieler, & Ewers, 2009; reviewed by Köhler & Dobrindt, 2011; Siqueira et al., 2009). Two new animal pathogenic subgroups have recently been proposed: mammary pathogenic (MPEC) (Shpigel, Elazar, & Rosenshine, 2008) and endometrial pathogenic *E. coli* (EnPEC) (Sheldon et al., 2010, reviewed by Köhler & Dobrindt, 2011). UPEC strains are associated with UTIs like cystitis and pyelonephritis in humans (Bélanger et al., 2011; reviewed by Bien et al., 2012; Johnson, Owens, Gajewski, & Kuskowski, 2005a), dogs and cats (Chen et al., 2003; Hagman & Kühn, 2002; Johnson, Kaster, Kuskowski, & Ling, 2003a; Siqueira et al., 2009) as well with pyometra in dogs (Chen et al., 2003). UPEC isolated from dogs, cats and humans are in some cases phylogenetically close related (Bélanger et al., 2011; Guardabassi, Schwarz, & Lloyd, 2004; Johnson et al., 2003a) and have a similar virulence gene profile (Johnson, Stell, & Delavari, 2001a). *E. coli* strains involved in cases of canine pyometra displays great similarity with those involved in canine UTIs, probably because in both cases bacteria are originated from the host's vaginal or intestinal flora (Chen et al., 2003; Hagman & Kühn, 2002).

In cattle, ExPEC are responsible for metritis, endometritis and mastitis. (Bélanger et al., 2011). *E. coli* is the most prevalent bacterium isolated during the first week postpartum from the uterus of cows that developed puerperal uterine infection (Mateus, Lopes Da Costa, Bernardo, & Robalo Silva, 2002; Sheldon, Noakes, Rycroft, Pfeiffer, & Dobson, 2002). Infection of the endometrium with Gram-negative *E. coli* is the first step in the disease process for developing uterine disease in cattle, preceding infection by the other bacteria such as *Trueperella pyogenes* and Gram-negative anaerobes such as *Fusobacterium necrophorum* and *Prevotella melaninogenicus* (Sheldon et al., 2002). However, the association between *E. coli* virulence traits and the development of puerperal or clinical metritis in the cow is controversial (Bicalho, Machado, Oikonomou, Gilbert, & Bicalho, 2012; Sheldon et al., 2010; Silva et al., 2009a).

## 2.1.Virulence Factors

Commensal and pathogenic bacteria typically differ with respect to phylogenetic background and virulence profiles (Johnson & Stell, 2000b). The number of ExPEC VFGs on a strain is proportional to its pathogenic potential (Picard et al., 1999). The ExPEC *E. coli* carry virulence determinants, like adhesins, toxins, fimbriae, iron acquisition factors, extracellular lipopolysaccharides, capsule and serum resistance, associated or not in pathogenicity-associated islands (PAIs). Some of these VFGs contribute to fitness during asymptomatic intestinal colonization in the host in which, eventually, given the right conditions, can lead to an extra-intestinal infection (reviewed by Antão et al., 2009; Diard et al., 2010; Oelschlaeger, Dobrindt, & Hacker, 2002). Some strains of *E. coli* can diverge from their commensal cohorts, taking on a more pathogenic nature through the acquisition of specific VFs via DNA horizontal transfer of transposons, plasmids, bacteriophages and PAIs, which increased bacteria ability to adapt to new niches and cause a broad spectrum of diseases (reviewed by Bien et al., 2012; Escobar-Páramo et al., 2004). Infection implies adhesion to host cells, colonization or internalization, multiplication and release of bacterial products that leads to infection and dissemination to other tissues or to persistence (Azawi, 2008;reviewed by Bien et al., 2012). The balance between the immune system of the host and the presence and expression of virulence factors of the bacteria determines the barrier between commensalism and virulence (Picard et al., 1999).

### 2.1.1. Adhesins

Bacterial adhesion to host cells is a crucial step for the establishment of infection. Bacterial adhesins contribute to virulence by directly triggering host and bacterial cell signaling pathways, facilitating the delivery of bacterial products to host tissues, and promoting bacterial invasion (reviewed by Bien et al., 2012)

The primary bacterial adherence factors are filamentous adhesive organelles known as fimbriae (pili) typically presented on bacterial surface or as afimbrial anchored within the bacterial outer membrane (Bower, Eto, & Mulvey, 2005). Fimbriae include type 1 fimbriae, present in more than 90% of all *E. coli* strains, P fimbriae presents in 40% - 60% of all *E. coli* strains and S fimbriae presents in 30% - 60% of all *E. coli* strains. The

afimbrial adhesins were only present in 0% - 12,5% of all *E. coli* strains (Blanco et al., 1997; Miyazaki et al., 2002). The best characterized fimbriae are able to agglutinate erythrocytes and this agglutination can be classified in mannose-resistant hemagglutination (MRHA) (S, P fimbriae and afa adhesins) or in mannose-sensitive hemagglutination (MSHA) (type I fimbriae), depending whether D-Mannose can inhibit the hemagglutination (Blanco et al., 1997; Van Den Bosch, Verboom-Sohmer, & Postma, 1980).

Type 1 fimbriae are highly conserved and commonly expressed by both commensal and pathogenic *E. coli* strains. This fimbriae is encoded by *fim* gene cluster, mediate adhesion to mannosides, are classified as MSHA and enable the bacteria to colonize a variety of host's epithelial surfaces (reviewed by Antão et al., 2009; reviewed by Johnson, 1991; Klemm, 1986). The expression of type 1 fimbriae is phase variable, which is associated with the inversion of a 314-bp DNA fragment located upstream to the *fimA* gene containing the *fimA* promoter (Klemm, 1986). Bacteria shift between a fimbriate (ON) and non-fimbriate state (OFF) that is controlled by the products of two regulatory genes, *fimB* and *fimE* (Klemm, 1986; Bergsten et al., 2005; Wullt, 2003). Moreover, differences in *fim* switching between UTI and commensal isolates or between cystitis and pyelonephritis isolates support the idea that regulation of *fim* expression may influence both overall pathogenicity and anatomical site tropism (Johnson & Russo, 2005b).

Type 1 fimbriae act as virulence factors in the human and murine urinary tract and improve bacterial attachment to the mucosa bladder through FimH adhesin (reviewed by Bien et al., 2012; Pizarro-Cerdá & Cossart, 2006; Ragnarsdóttir, Lutay, Grönberg-Hernandez, Köves, & Svanborg, 2011). Type 1 fimbriae mediates not only the adhesion but also facilitate the invasion and internalization of uroepithelial cells and trigger TLR4 signaling pathway (Pizarro-Cerdá & Cossart, 2006; Ragnarsdóttir et al., 2011).

Although found in several ExPEC strains (Bélanger et al., 2011), FimH plays an important role in UTIs caused by UPEC and so it has been tested as a vaccine candidate (reviewed by Antão et al., 2009). In canine pyometra, it was demonstrated that *E. coli* binding to endometrial epithelium is facilitated by FimH ( Krekeler et al., 2012).

Similarly to type 1 fimbriae, P fimbriae genes are commonly found in *E. coli* isolates from canine pyometra (Chen et al., 2003; Siqueira et al., 2009).These fimbriae are encoded by pyelonephritis-associated pili genes cluster *(pap* gene cluster), that contain 11 genes

which 6 encodes to fimbriae structural proteins, typically found on the chromosome of strains isolated from human urinary tract infections (Bergsten et al., 2005; Wiles, Kulesus, & Mulvey, 2008). P fimbriae mediates attachment through PapG adhesion located at the tip of the fimbria, by binding to a glicolipid receptor that contains a digalactoside (Galα1-4Gal) core linked by a β-glucose (Glc) residue to a ceramide group that anchors the receptor in the membrane (reviewed by Antão et al., 2009; Bergsten et al., 2005; Ragnarsdóttir et al., 2011). P fimbrial expression also undergoes phase variation (Bergsten et al., 2005). PapG adhesin molecule can be found in three molecular variants, PapG I, II and III, encoded by three different alleles of the *papG* gene. *papG* allele II is associated with human pyelonephritis and bacteremia isolates (Johnson et al., 2000a; Lane & Mobley, 2007) and *papG* allele III is associated with women and children cystitis isolates (Johnson et al., 2000a; Wiles et al., 2008), and with canine pyometra isolates (Chen et al., 2003). More than 80%, 60% and 20% of all pyelonephritis, cystitis and faecal *E. coli* strains, respectively, express P fimbriae (Wullt, 2003). P fimbriae and type 1 fimbriae activate different intracellular signaling pathways: P fimbriated bacteria preferentially activate TLR4/Toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF) signaling while type 1 fimbriae trigger TLR4 responses mainly involving myeloid differentiation primary response 88 (MyD88) signaling (Wullt, 2003; Yadav et al., 2010). The role of the P fimbriae in binding of *E. coli* to the canine endometrium is still unknown. However, more recently it was demonstrated that *E. coli* is able to fully compensate for the loss of two of its three known adhesin genes (*fimH*, *papGIII* and *sfa*), without a significant reduction in bacterial binding to canine endometrium. To obtain a significant decrease in binding to the endometrium it was necessary to inactivate all three known adhesin genes. This suggests that adhesins have functionally redundant properties (Krekeler et al., 2013).

Curli fibres are thin surface structures that are involved in biofilm formation and binds to fibronectin, laminin, plasminogen and to major histocompatibility complex (MHC) class I molecules (Gophna et al., 2001; Robinson, Ashman, Hultgren, & Chapman, 2006). Curli fibres (csg) are encoded on the *csg* gene cluster consisting of two different transcribed operons, one which encodes de *csgB*, *csgA* and *csgC* genes while the other encodes *csgD*, *csgE* and *csgG* genes and are expressed by pathogenic and non-pathogenic *E. coli* isolates, (reviewed by Antão et al., 2009; Gophna et al., 2001; Robinson et al., 2006).

S fimbriae adhesins recognize receptors containing sialic acid sugar moieties and have the capacity to agglutinate human and bovine erythrocytes (reviewed by Johnson, 1991; Khan et al., 2000; Marre, Kreft, & Hacker, 1990). Morphologically, S fimbriae are similar to type 1 or P fimbriae and were reported to be the most frequently found in *E. coli* strains associated with meningitis and neonatal sepsis although it was also detected in some pyelonephrogenic *E. coli* strains (reviewed by Antão et al., 2009). The *sfa* gene cluster consists of several subunits (*sfaA, B, C, G, H* and *S*) and *sfaS*, the minor subunit of S fimbriae was identified as the sialic acid-binding adhesion (reviewed by Antão et al., 2009; Prasadarao, Wass, Hacker, Jann, & Kim, 1993) and its expression is dependent on several environmental conditions like temperature, osmolarity and the presence of glucose (Prasadarao et al., 1993). *Sfa* is also an adhesin frequently detected in *E. coli* isolates associated to canine pyometra (Chen et al., 2003).

S fimbriae are genetically and immunologically related with F1C fimbriae (fimbriae encoded by the *foc* operon) (reviewed by Johnson, 1991; Marre et al., 1990). However, although contributing to adhesive properties of UPEC strains, F1C fimbriae (*foc*) confer no haemagglutination to erythrocytes from humans, oxen, horses, guinea-pigs or chickens (reviewed by Johnson, 1991; Khan et al., 2000; Riegman et al., 1990).

The first determinant to be identified that encodes for afimbrial adhesins was *afa-1* operon (Labigne-Roussel et al., 1984). Since then other genes of *afa* operon has been described. There are at least four different *afa* operons, *afa-1*, *afa-2*, *afa-3* and *afa-4* which encode for AFA-I, AFA-II, AFA-III and AFA-IV adhesins, respectively (Servin, 2005). Afimbrial adhesins also named MRHA adhesins agglutinate human erythrocytes in the presence of D-mannose (reviewed by Antão et al., 2009). Other related operons have also been reported including the *dra* operons detected in uropathogenic isolates (Nowicki et al., 1987; Pham et al., 1997). *afa* and *dra* operons have very similar genetic organization and are closely related at DNA level. Some gene subtypes encode adhesins named afa/dr adhesins that can encode for both afimbrial (such as AfaE-I and AfaEIII) and fimbrial (such as F1845 and Dr) adhesive structures on the bacterial surface (reviewed by Le Bouguénec & Servin, 2006). Dr fimbriae have been shown to bind to type IV collagen and decay-accelerating factor (DAF) of the basement membranes of human and canine kidneys, Bowman's capsule and bladder epithelium (reviewed by Bien et al., 2012; Goluszko et al., 1997; Nowicki et al., 1990; Van Loy, Sokurenko, & Moseley, 2002). Dr

adhesin encoding operon dra (*draA, B, C*, *D* and *E*) is required for full expression of the mannose resistant haemagglutinin phenotype (Goluszko et al., 1997; Servin, 2005). Dr fimbriae have been found to be prevalent among APEC, UPEC and NMEC isolates but in a lower percentage as compared to type 1 fimbriae, P fimbriae and S fimbriae (reviewed by Antão et al., 2009).

### 2.1.2. Toxins

Toxins are important virulence factors that may induce an inflammatory response and were often used to categorize ExPEC isolates (Marrs, Zhang, & Foxman, 2005) The toxins most frequently associated with UPEC *E. coli* strains are α-haemolysin (HlyA) and cytotoxic necrotizing factor-1 (Cnf1). Both toxins promote permeabilization and destruction of host cells, disrupting the mucosal barrier and causing different extra-intestinal infections (Ragnarsdóttir et al., 2011; Siqueira et al., 2009).

*hly* operon is genetically linked to the locus encoding Cnf1 toxin (Dhakal & Mulvey, 2012) and both toxins seem to be delivered to target host cells primarily via outer membrane vesicles (Wiles et al., 2008). HlyA is a pore-forming exotoxin calcium dependent, belonging to the RTX (repeats-in-toxin) toxin family (Dhakal & Mulvey, 2012; Schmidt & Hensel, 2004), widespread among Gram-negative bacteria and associated with upper UTIs such as pyelonephritis (reviewed by Bien et al., 2012). HlyA production is controlled by the expression of four genes that constitute the *hly* operon (*hlyCABD*). The *hlyA* gene encodes to HlyA, which is activated by HlyC, prior its secretion and release from the outer membrane by a mechanism involving the HlyB and HlyD proteins, respectively (reviewed by Johnson, 1991). The *hly* operon can be located on either a plasmid or on the chromosome (reviewed by Johnson, 1991; Schmidt & Hensel, 2004). Plasmid and chromosomal *hly* regions differ with respect to flanking and regulatory sequences and to the precise sequence of hlyA (reviewed by Johnson, 1991). At high concentrations, HlyA has a pore-forming activity in the membrane of erythrocytes, nucleated host cells and immune cells such as granulocytes and monocytes (Schmidt & Hensel, 2004) leading to cell destruction and osmotic lysis. This process may allow extra-intestinal pathogens like UPEC to better cross mucosal barriers, facilitating the release of nutrients and other factors like iron that are essential for bacteria growth and survival

(reviewed by Bien et al., 2012; Martínez-Martínez, Fernández, & Perea, 1999; Wiles et al., 2008). At low concentrations, HlyA can induce apoptosis of target host cells, including neutrophils, T lymphocytes, and renal cells, and promote the exfoliation of bladder epithelial cells (reviewed by Bien et al., 2012). HlyA has also been shown to induce $Ca^{2+}$oscillations in renal epithelial cells, resulting in increased production of IL-6 and interleukin-8 (IL-8) (reviewed by Bien et al., 2012). HlyA is encoded by 50% of UPEC isolates and its expression is associated with increased clinical severity in UTI patients (Wiles et al., 2008). In some strains, hemolysin production is suppressed in high-iron conditions and enhanced in low-iron conditions. Hemolytic activity is maximal in the supernatants of log-phase cultures of hemolytic strains and declines as cultures enter stationary phase (reviewed by Johnson, 1991).

Cnf1 is a chromosomally encoded UPEC toxin (Rippere-Lampe & O'Brien, 2001), that interferes with polymorphonuclear phagocytosis and promotes apoptosis of bladder epithelial cells, stimulating their exfoliation and increase bacterial access to host tissue (reviewed by Bien et al., 2012; Wiles et al., 2008). The toxicity of this protein is assigned to its ability to activate the Rho family GTPases that promote host cells membrane ruffling, actin stress fibers and DNA replication in the absence of cell division, a phenomenon that results in enlarged, multinucleated cells (Kouokam et al., 2006; Rippere-Lampe & O'Brien, 2001; Schmidt & Hensel, 2004; Wiles et al., 2008). Cnf1 also promotes changes in host cell gene signaling pathways involving certain nuclear transcription factors (Y. C. Smith, Rasmussen, Grande, Conran, & O'Brien, 2008). Cnf1 facilitate the dissemination and persistence of UPEC within the urinary tract and are involved in kidney invasion (reviewed by Bien et al., 2012; Wiles et al., 2008).

Some studies with *E. coli* mutants for *hlyA* and *cnf1* in mouse model of ascending UTI conclude that these two proteins may be responsible for the signs and symptoms of cystitis in humans (Marrs et al., 2005; Y. C. Smith et al., 2008). *hlyA* and *cnf1* are also virulence factor genes frequently detected in *E. coli* isolates from canine pyometra (Chen et al., 2003; Ghanbarpour & Akhtardanesh, 2012; Siqueira et al., 2009).

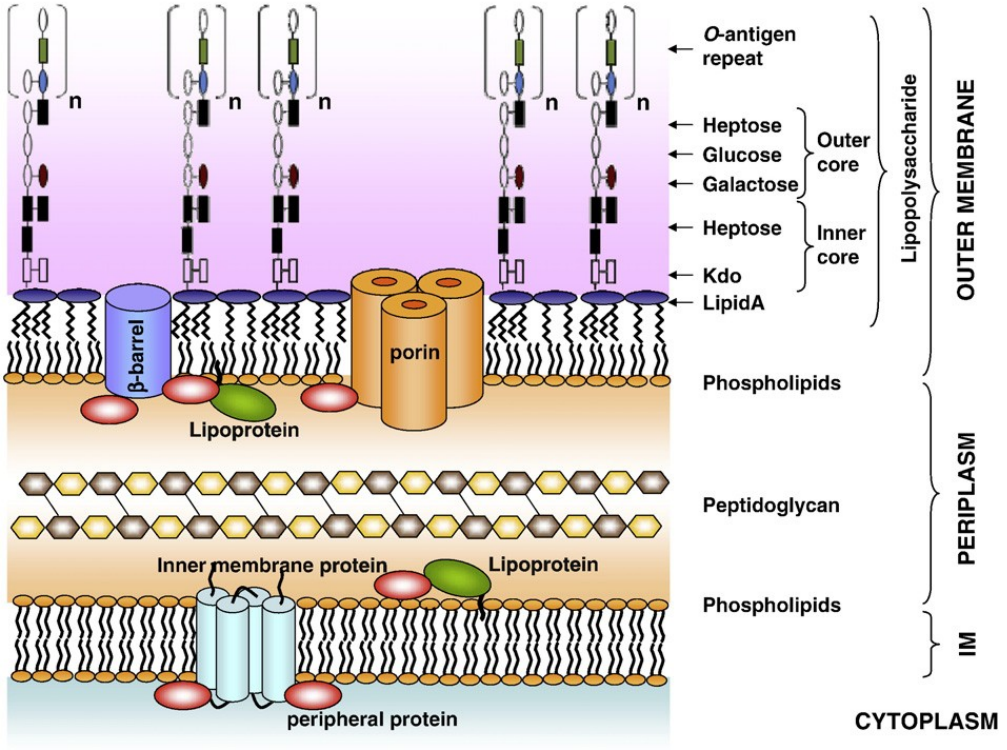
#### 2.1.2.1. Lipopolysaccharide

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria and acts as a potent endotoxin inducer of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-1 and interleukin-12 (IL-12) and inflammatory substances such as prostanoids, leukotrienes and nitric oxide in monocytes, macrophages, leucocytes, dendritic cells (DCs), epithelial and stromal cells (Ulevitch & Tobias, 1995; Van Amersfoort, Van Berkel, & Kuiper, 2003; Werling & Jungi, 2003; Yamamoto & Akira, 2005).

LPS consists of three parts: lipid A, a core oligosaccharide and O side chain (Lu, Yeh, & Ohashi, 2008; Wang & Quinn, 2010), works as a glycolipid complex essential for bacterial viability (Sperandeo, Dehò, & Polissi, 2009; Van Amersfoort et al., 2003) (Figure 1). Lipid A is the most essential and highly conserved hydrophobic portion of LPS and it is synthesized at the cytoplasmic side of the inner membrane (IM) and translocated to the outer leaflet of the outer membrane (OM) (Sperandeo et al., 2009; Wang & Quinn, 2010). It is responsible for the majority of the complications associated with Gram-negative bacterial infection such as endotoxin shock (reviwed by Akira, Uematsu, & Takeuchi, 2006; Sperandeo et al., 2009; Van Amersfoort et al., 2003). The second part of LPS molecule is the core oligosaccharide which connects to the lipid A through the inner core and to the O-antigen repeats through the outer core (Wang & Quinn, 2010). The third part of LPS molecule, consists of common sugars that provide the attachment site for the O-polysaccharide chain (Sperandeo et al., 2009; Van Amersfoort et al., 2003).

Several unique structural features of LPS contribute to the effective permeability barrier function of the OM. As a result, Gram-negative bacteria are protected from many toxic compounds such as bile salts, detergents, antibiotic and antimicrobial peptides (Sperandeo et al., 2009). Colonies with LPS molecules containing O-antigen are denoted S-LPS and have a smooth (S) appearance on agar plate whereas bacteria express an O-antigen-lacking LPS have a rough (R) appearance (Van Amersfoort et al., 2003). There are over 160 distinct O antigens in *E. coli* (Drummelsmith & Whitfield, 1999).

**Figure 1 - Structure of *Escherichia coli* cell envelope. *In* (Sperandeo et al., 2009)**



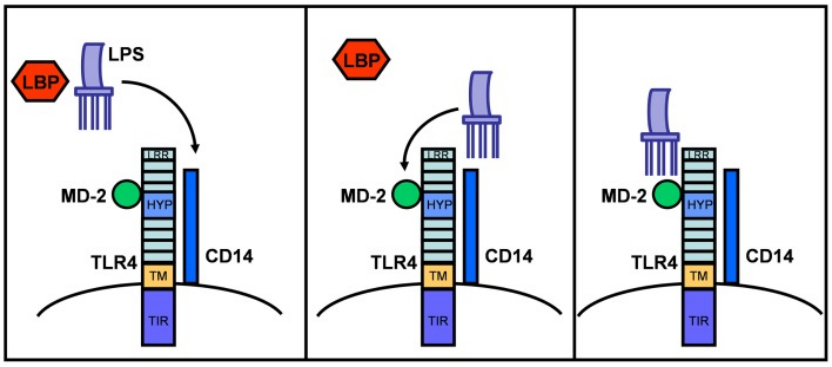

LPS is a complex glycolipid that can be structurally divided in three parts: lipid A, the hydrophobic moiety that anchors LPS to the outer membrane (OM), the oligosaccharide region named core, and the O-antigen polysaccharide chain. The core oligosaccharide of LPS can be divided into inner core, composed of Kdo (3-deoxy-D-manno-oct-2- ulosonic acid) and heptose, and outer core, which exhibits a greater structural diversity and provides the attachment site for the O-polysaccharide chain

LPS is recognized by LPS-receptor complex CD14, TLR4, LPS binding protein (LBP) and myeloid differentiation factor-2 (MD-2) (Werling, Jann, Offord, Glass, & Coffey, 2009; Werling & Jungi, 2003).

LPS released from Gram-negative bacteria associates with LBP via lipid A (Ulevitch & Tobias, 1995). LBP is an acute-phase protein present in the bloodstream, that mediates the transfer of LPS to CD14, a protein expressed on the surface of inflammatory (monocytes, macrophages, polymorphonuclear leukocyte (PMN) and B cells) and non-inflammatory cells, like liver parenchymal cells, gingival fibroblasts and bovine stromal and epithelial endometrial cells (reviewed by Akira et al., 2006; Herath et al., 2006; Van Amersfoort et al., 2003). LPS is then transferred to MD-2, which associates with the extracellular

portion of TLR4, followed by oligomerization of TLR4 (reviewed by Akira et al., 2006) (Figure 2). Furthermore, MD-2 on its own is produced in vast amounts within the host and acts as an opsonin increasing the phagocytosis on Gram-negative bacteria (Werling et al., 2009).

**Figure 2 - LPS sensing via LBP to CD14/MD-2/TLR4 receptor complex. *In* (Vaure & Liu, 2014)**




TLR4 consists of an extracellular domain with leucine-rich repeats (LRR), a hypervariable domain (HYP), a transmembrane domain (TM), and a cytoplasmic domain with a highly conserved TIR-domain. LBP mediates the transfer of LPS to CD14. LPS is then transferred to MD-2 and to the extracellular domain of TLR4, followed by oligomerization of TLR4.

### 2.1.3. Siderophores

Iron is essential for microorganisms grow and are involved in a variety of biological processes like reduction of oxygen for synthesis of adenosine triphosphate (ATP), DNA synthesis, oxygen transport, gene regulation and peroxide reduction (Garénaux, Caza, & Dozois, 2011; Krewulak & Vogel, 2008). In the mammalian host, free iron concentrations are very low and therefore bacteria have developed multiple strategies for stealing iron from the host (Braun, 2001; Wiles et al., 2008). Bacteria developed mechanisms to acquire iron from transferrin present in serum, lactoferrin of secretory fluids like lymph and mucosal secretions, ferritin present in cells and heme of hemoglobin and myoglobin present in erythrocytes (Braun, 2001; Doherty, 2007; Garénaux et al., 2011; Miethke & Marahiel, 2007). *E. coli* mechanisms of iron uptake can be divided into three main categories: (1) heme acquisition systems, (2) siderophore-based systems and (3) receptor-mediated iron acquisition from host proteins like transferrin/lactoferrin receptors (Krewulak & Vogel, 2008). All of these iron uptake pathways involve an outer membrane

receptor, a periplasmic binding protein (PBP) and an inner membrane ATP-binding cassete (ABC) transporter (Krewulak & Vogel, 2008). In the heme acquisition systems, iron can be directly scavenged from ferritin and hemoglobin through outer membrane transport heme acquisition systems such as Hma and ChuA (Gao et al., 2012; Garcia, Brumbaugh, & Mobley, 2011; Garénaux et al., 2011). Siderophore-based systems can be classified by their functional groups as catecholate (enterobactin (Ent); ferrienterobactin receptor (FepA), dihydroxy-benzylserine (Cir and Fiu), hydroxamate (aerobactin (IutA)), ferrichrome (FhuA), coprogen (FhuE), ferrioxamine B (FhuF) and citrate (FecA) (Guerinot, 1994; Miethke & Marahiel, 2007). Siderophores (iron carriers), that are low molecular weight molecules with high affinity and specificity for ferric iron (Andrews, Robinson, & Rodríguez-Quiñones, 2003), capture iron from environment and solubilize iron ($Fe^{3+}$) prior to transport (Andrews et al., 2003; Wiles et al., 2008). Since $Fe^{3+}$ are too large to pass through the porins, bacteria recover iron-bound siderophores through specific outer membrane receptors proteins that facilitate the transport of siderophore–iron complexes through the bacterial membrane and transport iron into the cytosol where it is released and concentrated (Andrews et al., 2003; Braun, 2001; Wandersman & Delepelaire, 2004; Wiles et al., 2008). The receptor-mediated iron acquisition from host proteins like transferrin and lactoferrin receptors occur via TbpB/TbpA and LbpB/LbpA proteins, respectively, since they are too large to pass through the bacterial outer membrane (Krewulak & Vogel, 2008).

*E. coli* have different combinations of iron acquisition systems and although siderophore and heme uptake systems contribute to the fitness of UPEC, the understanding of the role each iron system remains unknown (Hagan & Mobley, 2010). However work by Garcia and co-workers (2011) suggest that UPEC iron receptors provide both functional redundancy and niche specificity for this pathogen as it colonizes distinct sites within the urinary tract. Moreover, the major advantages of multiple iron uptake systems in UTI-associated *E. coli* are likely to reflect the ability to successfully compete for iron against both the host and other bacteria (Watts et al., 2012).

### 2.1.4. Other virulence factors

Pathogens have developed mechanisms to inhibit the TLR dependent host defense and to increase their fitness and virulence (Yadav et al., 2010). Inhibition of TLR signaling cascade by microbial TIR proteins is advantageous for microorganisms through the reduction of pro-inflammatory cytokine release, like TNF-α and IL-6 (Cirl & Miethke, 2010). A TIR domain-containing protein (TcpC) of UPEC (Cirl et al., 2008) is one of the factors involved in the suppression of the host innate immune response. TcpC inhibits TLR and Myd88 dependent signaling, as well as downstream effector functions through the binding to TLR4 and Myd88 via TIR domain interactions (Yadav et al., 2010). TcpC increases the severity of UTIs in humans and provided the first evidence that bacterial pathogens interfere with TLR signaling to survive and spread in the human host (Starcic Erjavec, Jesenko, Petkovsek, & Zgur-Bertok, 2010). In a murine urinary tract model, *tcpC*-positive CFT073 strain induced the formation of abscesses in the kidneys and increased the bacterial burden in the urine, suppressing innate host responses, enhancing bacterial persistence and tissue damage, compared with *tcpC*-deficient mutant (Cirl & Miethke, 2010; Yadav et al., 2010). Also, in a mouse model of UTI, the *tcpC* knockout strain had reduced virulence compared with the wild-type strain (Rana, Zhang, Spear, Atkins, & Byrne, 2012). *tcpC* gene is encoded on a novel genomic island (*serU* island) located next to the high-pathogenicity island (HPI) (Schubert et al., 2010). TcpC homologous sequences were present in about 40% of *E. coli* isolates from individuals with pyelonephritis, 20% of *E.coli* isolates from individuals with cystitis, 16% of *E. coli* isolates from individuals with asymptomatic bacteriuria (Cirl & Miethke, 2010; Starcic Erjavec et al., 2010) and only in 8% of commensal *E. coli* isolates (Starcic Erjavec et al., 2010). Strong statistical correlations were found between the presence of *tcpC* and B2 phylogenetic group (Erjavec et al., 2010).

Another virulence factor that has been associated with UPEC strains is theuropathogenic specific protein that is encoded by the bacteriocin gene (*usp*). This protein enhances the infectious potential of *E. coli* strains in a pyelonephritis mouse model (Zaw et al., 2013) suggesting that Usp may play a role in UPEC pathogenesis. It may be also involved in the pathogenesis of canine pyometra (Siqueira et al., 2009) as suggested in canine UTIs (Kurazono et al., 2003).

### 2.1.5. Pathogenicity islands

Many VFs of UPEC commonly occur together associated PAIs. PAIs are large blocks of chromosomal DNA (10 to 200 kb) containing virulence gene clusters (Boyd & Hartl, 1998), mostly located on bacterial chromosome but also in plasmids or bacteriophages (Hacker & Carniel, 2001; Hacker & Kaper, 2000; Picard et al., 1999). PAIs are often flanked by insertion sequence elements or transfer ribonucleic acid (tRNA) genes like *pheU, pheV, selC* and *leuX5* (Boyd & Hartl, 1998; Hacker & Kaper, 2000; Lloyd, Henderson, Vigil, & Mobley, 2009; Schmidt & Hensel, 2004) and are characterized by a GC content that differs from the host bacterial genome (Lloyd et al., 2009; Sabaté, Moreno, Pérez, Andreu, & Prats, 2006; Schmidt & Hensel, 2004).

PAIs have been spread among bacteria by horizontal transfer and supply a virulence benefit regarding the adaptation to niches incapable to be colonize by commensal bacteria (Johnson & Russo, 2005b; Lloyd et al., 2009; Oelschlaeger et al., 2002). The eight most studied PAIs described in UPEC are: PAI I and II in *E. coli* J96, PAI I and II in *E. coli* CFT073, and PAI I–IV in *E. coli* 536 (Sabaté et al., 2006). The identification of new PAIs in UPEC strains will help to identify UPEC pathotypes and new targets for therapy. These UPEC PAIs have been also detected in *E. coli* strains causing bacteremia and neonatal meningitis (Bingen-Bidois et al., 2002; Johnson & Russo, 2002; Russo & Johnson, 2000) as also in commensal isolates from fecal origin (Johnson & Russo, 2002; Sabaté et al., 2006). In human *E. coli* isolates, PAIs were detected in a substantial percentage of commensal isolates (40%), although this was much lower than the percentage detected in UPEC isolates (93%) (Sabaté et al., 2006).

ExPEC strains often contain multiple PAIs, each with a different combination of VFs, which sometimes results in a strain having multiple copies of a particular VF (Johnson & Russo, 2005). Moreover, as the same VF can be encoded by different PAIs, the isolates with multiple PAIs can lose one PAI and acquire new VFs with no effect on phenotype. In commensal isolates like intestinal strains, these DNA segments may act as fitness islands or ecological islands rather than as PAIs (Diard et al., 2010; Hacker & Kaper, 2000). These islands may also carry genes encoding factors that confer resistance to antimicrobial substances (Hacker & Carniel, 2001).

### 2.1.6. Phylogenetic groups

*E. coli* strains fall into four main phylogenetic groups A, B1, B2 e D (Herzer, P. Inouye, S. Inouye & Whittam et al. 1990) which can be assessed by a polymerase chain reaction (PCR) based method directed to the genetic markers *chuA*, *yjaA* and the DNA fragment TspE4.C2 (Clermont, Bonacorsi, & Bingen, 2000) The extra-intestinal pathogenic strains harboring a high number of virulence related genes and subsequently a high virulence potential usually belong to groups B2 and D (Clermont et al.,, 2000; Diard et al., 2010; Escobar-Páramo et al., 2004; Johnson et al., 2001a; Le Gall et al., 2007; Picard et al., 1999) The commensal strains, harboring a lower number of virulence related genes and with a lower virulence potential belong to groups A and B1 (Johnson et al., 2001a; Johnson & Russo, 2002; Le Gall et al., 2007; Sabaté et al., 2006), whilst the intestinal pathogenic strains belong to groups A, B1 and D (Pupo, Karaolis, Lan, & Reeves, 1997)
The intestinal flora may act as a reservoir of phylogenetic group B2 isolates, since B2 *E. coli* isolates seem to have a special privileged role in eliciting urinary tract infection (Sabaté et al., 2006). Recently, in order to increase the discrimination power of *E. coli* population analyses, it has been proposed the use of subgroups $A_0$, $A_1$, B1, $B2_2$, $B2_3$, $D_1$ and $D_2$, that are determined by the combination of the genetic markers (Escobar-Páramo et al., 2006).

## 2.2. Antimicrobial resistance

Companion and food animals may act as reservoirs of antimicrobial-resistant ExPEC that can be transmitted to humans (or *vice versa*) by direct contact (petting, licking, physical injuries) or indirectly, via food chain (Guardabassi et al., 2004; Johnson et al., 2001b; Marshall & Levy, 2011; Ramchandani et al., 2005; Stenske et al., 2009). An example is the similarities in phylogenetic, pathotypic and genotypic profile patterns among canine *E. coli* isolates and *E. coli* isolates from women with cystitis, pyelonephritis or urosepsis (Johnson et al., 2001b; Stenske et al., 2009). The use of antimicrobials in agriculture, farm management and in veterinary medicine including livestock and companion animals favours dissemination of antimicrobial resistance in animals and in the environment, leading to increased resistance to antimicrobials in bacteria colonizing humans (Bélanger

et al., 2011). In fact, close association exists between the use of antimicrobial agents in animals and the levels of resistance observed (Maynard et al., 2004). Since the same classes of antimicrobials are frequently used in veterinary and human medicine (Bélanger et al., 2011; Guardabassi et al., 2004; Maynard et al., 2004) this may have significant implications for the selection of antimicrobial resistance among human and animal pathogens (Johnson et al., 2001b).

To choose an appropriate antibiotic to treat an uterine infection it is essential to know the minimum inhibitory concentrations (MICs) of the antimicrobial agents used (Sheldon, Bushnell, Montgomery, & Rycroft, 2004b).

The first line of antimicrobials used in the treatment of common UTIs and pyometra are amoxicillin, amoxicillin and clavulanic acid, cefotaxime, fluoroquinolones and sulphonamides/trimethoprim (Guardabassi et al., 2004). Resistance to broad-spectrum antimicrobials, such as the fluoroquinolones and cephalosporins, among pathogenic *E. coli* isolates from companion animals is not surprising since veterinarians commonly use these drugs as first-line therapeutics in the treatment of certain infections in pet animals (Lanz, Kuhnert, & Boerlin, 2003; Sanchez et al., 2002).

In cattle, antimicrobial agents have been used to treat uterine bacterial infections like postpartum metritis and endometritis. During the first three weeks postpartum, around 40% of dairy cows develop metritis and antibiotics as ceftiofur and tetracycline are often systemically administered. In contrast with the high-level of resistance against tetracycline (Sheldon et al., 2004b), low levels of resistance of *E. coli* to cefquinome and ceftiofur were observed which might suggest that cephalosporin are more appropriate for uterine infection treatment in cattle (Chenault et al., 2004; Sheldon et al., 2004b; Sheldon, Rycroft & Zhou, 2004a). Endometritis is diagnosed after three weeks of postpartum and intrauterine administration of cephapirin is usually done (Sheldon & Noakes, 1998). *E. coli* antimicrobial drug resistance is strongly related to phylogenetic grouping (Johnson, Kuskowski, Owens, Gajewski, & Winokur, 2003b). It seems that there is a possible trade-off between resistance and virulence in ExPEC: in general, phylogenetic group B2 strains are more antibiotic-susceptible. In contrast, antibiotic-resistant strains, are more frequently from groups and A, B1 and D (Cooke, Smith, Kelleher, & Rogers, 2010; Houdouin et al., 2006; Johnson, Kuskowski, O'Bryan, Colodner, & Raz, 2005c).

However, Song et al. (2009) reports that more than half of fluoroquinolone-resistant *E. coli* isolates involved in UTIs were from group B2.

Antimicrobial resistance genes are located on plasmids, transposons, and integrons which are known to facilitate their distribution (Maynard et al., 2004) and enables their transfer among a variety of bacterial species (Marshall & Levy, 2011).

## 3. Immune system

The immune system integrates two components: the innate immunity and the adaptive immunity. Both components recognize invading microorganisms as non-self (Takeda & Akira, 2005). The innate immune system is nonspecific but evolutionally conserved, and not only provides the first line of defense against microorganisms but also provides the biological context that elicits the adaptive immune system to initiate a response (reviewed by Luster, 2002). The innate immune system is based on natural antimicrobial peptides (NAPs) and on specialized pattern recognition receptors (PRRs) which include the Toll-like receptors family (TLRs) and the nucleotide-binding oligomerization domain proteins (NOD-like receptors (NLRs)). The PRRs of the host recognize conserved microbial components of various bacterial, fungal and viral pathogens, such as lipopolysaccharides, peptidoglycans, flagellin, bacterial DNA and viral double stranded RNA (dsRNA), known as pathogen associated molecular patterns (PAMPs) (Albiger, Dahlberg, Henriques-Normark, & Normark, 2007; Chotimanukul & Sirivaidyapong, 2011; Yamamoto & Akira, 2005). The adaptive immune response is more specific and has a long-lasting memory and is elicited about 4-7 days post-infection (Kawai & Akira, 2006; reviewed by Luster, 2002).

### 3.1. Innate immunity: Toll-like receptors

TLRs have been identified as having a key role in mediating innate immune system, through the detection of a wide range of pathogenic stimuli with subsequent activation of intracellular pathways associated with immune function (Aboussahoud et al., 2010; Lea & Sandra, 2007). TLRs are type I transmembrane proteins with a cytoplasmic region called

TIR domain that is involved in downstream signal transduction (Kannaki, Shanmugam, & Verma, 2011; Takeda & Akira, 2005). They are evolutionary conserved and homologous receptors are found in plants, insects, worms (*Caenorhabditis elegans*) and vertebrates (Albiger et al., 2007). So far, 13 members of TLRs have been identified in mammals, 10 in human and 13 in mice. TLR1-9 are homologues in human and mouse (Albiger et al., 2007; Yu, Wang, & Chen, 2010). High homology between TLR sequence of human, mouse and domestic animal species suggests the conserved role of TLRs in immunity across the phylogenetic map (Kannaki et al., 2011).

TLRs are expressed by antigen presenting cells (APC) involved in the first line of host defense such as macrophages, dendritic cells, neutrophils, T and B cells but also on non-myeloid cells like epithelial cells, fibroblasts and endothelial cells (reviewed by Akira et al., 2006; Albiger et al., 2007; Chotimanukul & Sirivaidyapong, 2011; Imler & Hoffmann, 2001; Rana et al., 2012). TLRs can also be grouped into two subfamilies based on their cellular localization and ligand specificity: TLRs expressed on cell surface that recognize several compounds from microbes (TLRs 1, 2, 4, 5, 6, 10 and 11) and TLRs expressed on the membranes of intracellular organelles such as endosomes that recognize microbial nucleic acids or nucleotides derivates (TLRs 3, 7, 8 and 9) (Kannaki et al., 2011; Miyake, 2007; Rana et al., 2012; Yu et al., 2010).

TLR2 and TLR4 are the best characterized with respect to innate immune responses. TLR2 recognizes several microbial components such as lipoteichoic acid (LTA) from Gram-positive bacteria, lipoproteins/lipopeptides and peptidoglycans from Gram-positive and Gram-negative bacteria, lipoarabinomannan from mycobacteria, zymosan from yeast and fungi, glycosylphosphatidylinositol anchors from *Trypanosoma cruzi*, a phenol-soluble modulin from *Staphylococcus epidermis* and glycolipids from *Treponema maltophilum* (Kannaki et al., 2011; Takeda & Akira, 2005; Yu et al., 2010). Moreover, TLR2 recognizes also LPS preparations from non-enterobacteria such as *Leptospira interrogans*, *Porphyromonas gingivalis* and *Helicobacter pylori* that are structurally different from typical LPS of Gram-negative bacteria (Takeda & Akira, 2005). This capacity of TLR2 to recognize several ligands can be explained by its ability to form heterophilic dimers with TLR1, that recognize triacylated lipoproteins; and with TLR6, that recognizes diacylated lipoproteins and also fungal-derived components (Aboussahoud et al., 2010; Albiger et al., 2007; Takeda & Akira, 2005; Werling & Jungi, 2003). TLR4 is

the most extensively studied PRR and identifies Gram-negative bacteria by recognizing lipid A of LPS, a very potent immunostimulator (Kannaki et al., 2011). The recognition of LPS by TLR4 seems to be dependent on formation of a complex formed by the MD2, membrane-bound CD14 (mCD14) and LPB (Miyake, 2007; Yu et al., 2010). Also, TLR4 has been shown to be involved in the recognition of endogenous ligands, such as heat shock proteins (HSP60 and HSP70), the extra domain A of fibronectins, oligosaccharides of hyaluronic acid, heparin sulfate and fibrinogen (Takeda & Akira, 2005; Werling & Jungi, 2003). TLR3 recognizes viral dsRNA generated during virus replication and a synthetic analogue of dsRNA, polyinosine-deoxycytidylic acid (poly I:C) that is a potent inducer of type I interferons (IFNs) (reviewed by Akira et al., 2006; Yamamoto & Akira, 2005). TLR5 recognizes bacterial flagellin, a monomeric constituent of bacterial flagella (Kannaki et al., 2011; Takeda & Akira, 2005; Werling & Jungi, 2003). TLR7 and TLR8 are involved in recognition of single stranded RNA (ssRNA) (Kannaki et al., 2011; Takeda & Akira, 2005). TLR9 is a receptor for unmethylated CpG DNA, with motifs found in prokaryotic genome and absent in eukaryotic genomes (Albiger et al., 2007; Kannaki et al., 2011; Takeda & Akira, 2005). TLR10 is able to homodimerize or heterodimerize with TLR1 and TLR2 (Yu et al., 2010) but its ligand is currently unknown (Rana et al., 2012; Yu et al., 2010). TLR11 has been shown to be expressed in kidney and bladder epithelial cells and mediate resistance to infection by UPEC strains in mouse (reviewed by Kawai & Akira, 2005; Yamamoto & Akira, 2005). In humans, TLR11 protein is nonfunctional and became lost through evolution (Aboussahoud et al., 2010; reviewed by Kawai & Akira, 2005; Takeda & Akira, 2005; Yu et al., 2010).

### 3.2. TLRs signaling pathways

The cytoplasmic TIR domain of the TLRs interacts with TIR-domain-containing adaptors, such as MyD88, TIR-containing adaptor protein (TIRAP/MAL), TIR-domain-containing adaptor molecule 1 (TICAM1, also known as TRIF) and TIR-domain-containing adaptor molecule 2 (TICAM2, also known as TRAM). PAMP binding to the respective receptor results in the activation of the MyD88-dependent and/or MyD88-independent (TICAM1/TRIF) signaling pathways.

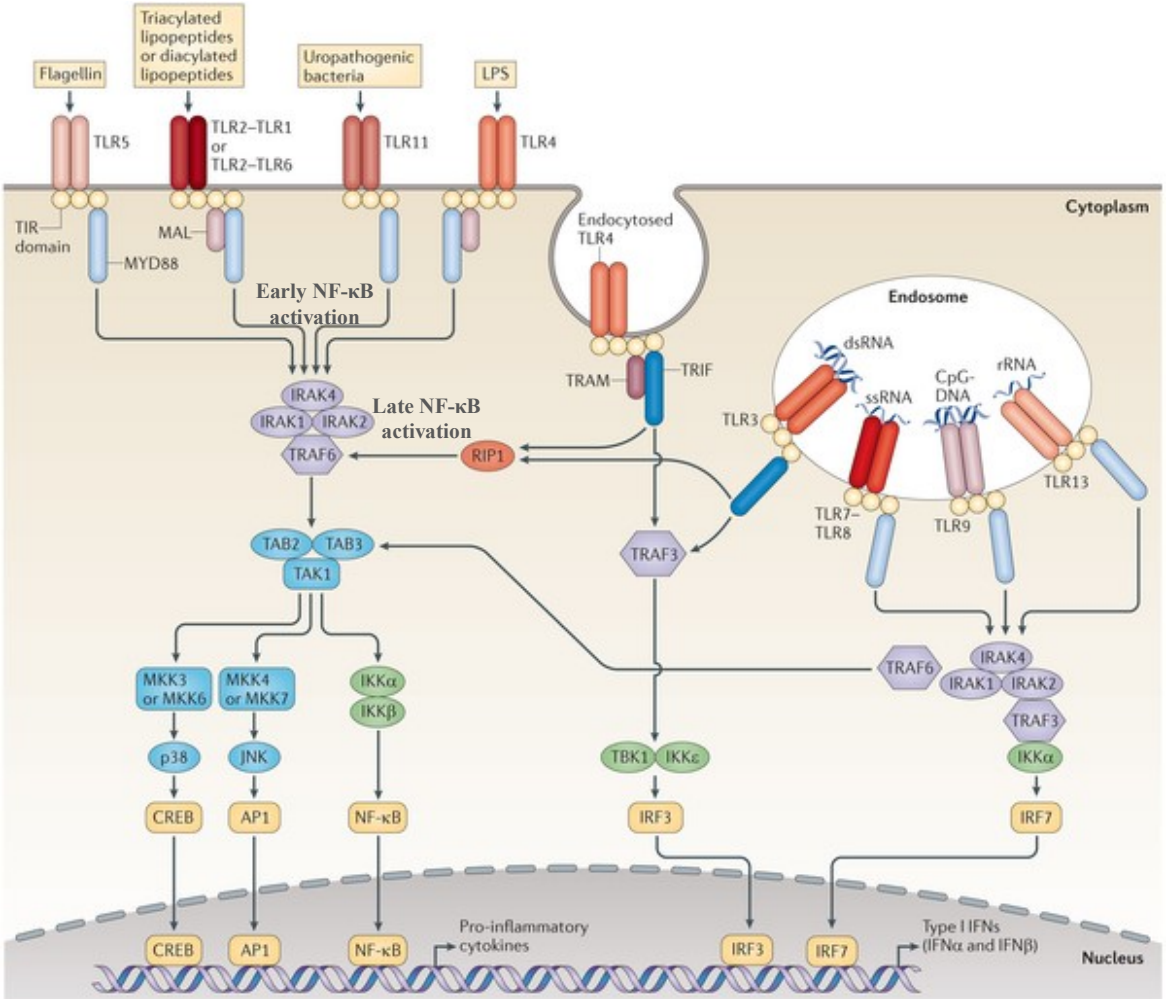
In both cases there is activation of nuclear factor kappa B (NF-κB) and activation of mitogen-activated protein (MAP) kinases (MAPKs) leading to the induction of genes that codifies for cytokines, chemokines, apoptosis regulating molecules and several transcription factors (Kannaki et al., 2011; Kumar, Kawai, & Akira, 2009; Yamamoto & Akira, 2005).

MyD88-dependent pathway is used by all TLRs except TLR3 (reviewed by Kawai & Akira, 2005; Rana et al., 2012) (Figure 3). TLR3 stimulation only activates TICAM1/TRIF signaling pathway and TLR4 stimulation activates both signaling pathways (MyD88-dependent and MyD88-independent) (Figure 3). Each TLR recruits a specific combination of adaptors to activate different transcription factors: the TIR domain of TLR1, TLR2, TLR4-TLR9 and TLR11 recruits MyD88 (MyD88-dependent pathway) that possesses a death domain in the N-terminal portion and a TIR domain in the C-terminal portion (Figure 3) (Yamamoto & Akira, 2005). Upon stimulation, MyD88 recruits IL-1 receptor-associated kinase (IRAK-1) and IRAK-4, which are activated by phosphorylation. TNF receptor-associated factor 6 (TRAF6) is recruited to the receptor complex, and activated by IRAK-1 that binds to the TRAF domain of TRAF6 (Figure 3). TRAF6 is a member of the tumor necrosis factor receptor (TNFR)-associated factor (TRAF) family that mediates cytokine signaling pathways. As a result, there is activation of the inhibitor of κ B kinase (IKK) enzyme complex and the MAPK pathway (Figure 3). The IKK complex phosphorylates the inhibitory IκBa protein, thus freeing the nuclear transcription factor nuclear NF-κB; triggering of the MAPK signaling pathway results in the activation of activator protein 1 (AP-1) (Figure 3). NF-κB and AP-1 enter the nucleus and activate transcription sites for a variety of genes, including acute phase proteins, inducible nitric oxide synthase (iNOS), coagulation factors, and pro-inflammatory cytokines and chemokines, such as TNF-α and ILs-1, 6, 8, and 12 (reviewed by Lewis et al., 2012). In the case of TLR2 and TLR4 signaling pathways, TIRAP/MAL is also required for the activation of the MyD88-dependent pathway (reviewed by Akira et al., 2006; reviewed by Kumar et al., 2009; Miyake, 2007). MyD88-independent signaling pathway leads to the activation of transcription factors like the late phase NF-κB, IFN regulatory factor 3 (IRF3) and MAP kinases which activates the transcription factor AP-1 and induce subsequent production of cytokines, type I IFN (IFN-α/β) and chemokines (Figure 3) (reviewed by Kawai & Akira, 2005; reviewed by Kumar et al., 2009; Rana et

al., 2012; Yu et al., 2010) (Figure 3). For IRF3 activation, TLR4 recruits TRAM adaptor protein which links the TIR domain of TLR4 to TRIF (Figure 3) (reviewed by Kawai & Akira, 2005; reviewed by Kumar et al., 2009; Rana et al., 2012; Yu et al., 2010). Upon stimulation, TRIF interacts with TBK1 and IKKi, which phosphorylates IRF3, allowing IRF3 to translocate into the nucleus and activate the IFN-β promoter (Figure 3). Among the nine IRF family members, IRF-3 and IRF-7 are essential for the induction of type I IFN production (reviewed by Akira et al., 2006). TLR7 and TLR9 ligands induce type I interferon secretion (mainly IFN-α) through activation of IRF7 and also induce inflammatory cytokines secretion through activation of NF-κB and AP-1 via MyD88-dependent pathway (Figure 3) (reviewed by Kumar et al., 2009). IRF7 is phosphorylated by IRAK1 and/or IKKα, translocate to the nucleus and induces transcription of IFN-α (reviewed by Kumar et al., 2009).

The collaboration between TLR family members and other PRRs such as NLRs and Rig-like helicases receptor (RLHs) is evident, playing an important role in innate immune response network to different invading organisms (Aboussahoud et al., 2010; reviewed by Kumar et al., 2009). The first NLR proteins identified, Nod1 and Nod2 recognize a wide range of ligands including bacteria, fungi, DNA or RNA viruses and endogenous ligands that activate NF-κB, stress kinases, IRFs, inflammatory caspases and autophagy (Dostert et al., 2008; reviewed by Kumar et al., 2009; Correa, Milutinovic & Reed, 2012). NLR signaling is very similar to the extracellular receptor TLR stimulation (Antosz & Osiak, 2013).

**Figure 3 – Mammalian TLR signaling pathways**




TLRs recognize several pathogen-associated molecular patterns and the TIR domain-containing adaptors such as MyD88, MAL, TRIF, and TRAM determine the TLR mediated signaling. MAL and MyD88 proteins are adaptors for the MyD88 dependent pathways, which leads predominantly to the production of pro-inflammatory cytokines. However, MyD88 could also be involved in the production of type I IFNs through the activation of endosomal TLRs. By contrast, TRIF and TRAM are the adaptors for the MyD88 independent pathway, which could activate the production of pro-inflammatory cytokines (activation of late NF-κB) or type I IFNs (activation of the IRF3 transcription factor). Adapted from (O'Neill, Golenbock, & Bowie, 2013)

### 3.3. Innate immunity in the uterus

Endometrial epithelial cells are known to be an efficient physical barrier to infection with the ability to modulate the recruitment and activity of immune cells of both innate and adaptive immune systems.

Toll-like receptors have been described in female reproductive tract of several mammalian species. Human female reproductive tract express TLR1 through TLR10, (Young et al., 2004; Nishimura & Naito, 2005) however, the transcription and expression of TLR2–6, TLR9 and TLR10 genes changes during the menstrual cycle (Aflatoonian et al., 2007; Schaefer, Desouza, Fahey, Beagley, & Wira, 2004). TLRs expression is higher during secretory phase of the cycle, when the progesterone level is high and when immune defenses are suppressed. Conversely, the oestrogen seems to have an inhibitory effect on the expression of TLRs in the endometrium (Aflatoonian et al., 2007; Hirata et al., 2007). The expression of TLR1 to TLR9 has been reported in human endometrial cell lines and in primary uterine epithelial cell cultures (Schaefer et al., 2004). In nonpregnant cattle, endometrium express TLR1 through TLR10 and purified populations of epithelial cells express TLR1 through TLR7 and TLR9, while stromal cells express TLR1-TLR4, TLR6, TLR7, TLR9 and TLR10 (Davies et al., 2008; Sheldon, Cronin, Goetze, Donofrio, & Schuberth, 2009). Similarly, TLR2 and TLR4 genes are transcribed in normal canine diestrous endometrium and up-regulated in the endometrium of bitches with pyometra. This up-regulation is probably induced by LPS and lipoproteins and is also associated with the high infiltration of leucocytes, mainly neutrophils in the endometrium in response to bacteria challenge (Hagman, Rönnberg, & Pejler, 2009; Silva et al., 2010; Zähringer, Lindner, Inamura, Heine, & Alexander, 2008). Chotimanukul & Sirivaidyapong (2011) reported the expression of TLR4 in the surface epithelium, glandular epithelium and stromal cells throughout the canine estrous cycle and also in cases of canine pyometra.

In the ovary, TLRs are involved in follicular development, ovarian follicle rupture and oocyte release and formation and regression of corpus luteum (Girling & Hedger, 2007; Liu, Shimada, & Richards, 2008). Furthermore, the TLRs are also involved in the immune response of cells from vagina, cervix and fallopian tubes against ascending infection (Amjadi, Salehi, Mehdizadeh, & Aflatoonian, 2014; Girling & Hedger, 2007), in normal

maternal-fetal interface pregnancy and in pregnancy-related disorders (reviewed by Abrahams et al., 2005; Amjadi et al., 2014; Challis et al., 2009; Girling & Hedger, 2007) and also involved in implantation mechanisms (reviewed by Abrahams et al., 2005; Patni et al., 2007).

Activation of TLR pathways can also stimulate the production of prostaglandins (PG) E and F by immune (Uematsu, Matsumoto, Takeda, & Akira, 2002) and endometrial cells (Herath et al., 2006). Pro-inflammatory stimuli, such as endotoxins released by Gram-negative bacteria, are potent stimulators of prostaglandin synthesis (Herath et al., 2006). In bitches with *E. coli* pyometra, an endometrial up-regulation of cyclooxygenase-2 (COX-2), prostaglandins E2 and F2α synthases (PGES and PGFS) gene transcription (Silva, Leitão, Ferreira-Dias, Lopes da Costa, & Mateus, 2009a) was observed, which was associated with a high endometrial concentration of $PGE_2$ and $PGF_{2\alpha}$ (Silva et al., 2010). $PGE_2$ is known for its immunosuppressive effect and the immunomodulatory role of PGE2 is observed on lymphocytes, monocytes/macrophages and PMN (Rocca and FitzGerald, 2002). The high uterine concentrations of PGE2 could further contribute to the suppressed activity of cellular immunity during diestrus (Silva et al., 2010). Plasma concentrations of PG-metabolite are elevated in pyometra bitches and provide a good indicator of endotoxin release since the concentrations are significantly correlated to the endotoxin levels and other hematological and chemistry parameters (Hagman, Kindahl, & Lagerstedt, 2006b).

### 3.4. Cytokines and Chemokines

Cytokines are soluble small polypeptide or glycoprotein mediators that regulates the immune and inflammatory responses and includes ILs, IFNs, growth factors and chemokines (reviewed by Tosi, 2005). Most cytokines are difficult to detect in serum because only small amounts are released to systemic circulation, since their producer cells are often adjacent to the target cells (Heinrich, Behrmann, Müller-Newen, Schaper, & Graeve, 1998). They exert autocrine, paracrine or endocrine actions, via specific cell surface receptors on target cells (Heinrich et al., 1998). Chemokines are small cytokine-like polypeptides involved in the migration of immune effector cells like neutrophils, monocytes/macrophages, dendritic and natural killer cells to the infection sites. The

chemokines are involved in many biological processes like angiogenesis, angiostasis, hematopoiesis, organogenesis, cell proliferation, lymphocyte polarization, apoptosis, tumor metastasis and host defense (reviewed by Esche, Stellato, & Beck, 2005; reviewed by Tosi, 2005). Pro-inflammatory cytokines such as IL-1, TNF-α, IFN, IL-4, IL-5, IL-6, IL-13 and IL-17, can be produced by epithelial cells, dendritic cells and macrophages as a consequence of TLRs activation (reviewed by Esche et al., 2005). Chemokines downstream of TLR activation include IL-8 (CXCL8) preferentially induced by TLR2 agonists, IP-10 (CXCL10) preferentially induced by TLR4 agonists like LPS and MIP-1α (CCL3), MIP-1β (CCL4) and RANTES (CCL5) induced by agonists of both TLR2 and TLR4 (reviewed by Luster, 2002; Re & Strominger, 2001).

Pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α regulate systemic response to microbial infection and tissue macrophages are the main source of these cytokines (Baer et al., 1998). The roles of IL-1 (IL-1α and IL-1β) in immunity includes the increase of lymphocyte responses and stimulation of acute-phase proteins (reviewed by Dinarello, 1994). Unlike pro-IL-1β, which has to be cleaved in order to be active as IL-1β, pro-IL-1α is biologically active and binds the IL-1R on adjacent cells, leading to secretion of cytokines such as IL-6 (Healy, Cronin & Sheldon, 2014). IL-6 is a pro-inflammatory cytokine that induces terminal differentiation of proliferating B cells to plasma cells, stimulates antibody secretion by plasma cells, promotes leucocyte recruitment, and enhances T-lymphocyte responses in secondary lymphoid organs (Schaefer et al., 2004). TNF-α is produced by neutrophils, lymphocytes, natural killer cells, endothelium, and mast cells and is a potent activator of neutrophils, mediating adherence and chemotaxis and is also the primary mediator of septic shock (reviewed by Commins, Borish, & Steinke, 2010). The control of pro-inflammatory responses to avoid excessive immune activation by microorganisms is dependent of anti-inflammatory mediators such as IL-10 and TGF (transforming growth factor)-β (Herath et al., 2009b). TGFβ is produced by different cell types, including eosinophils, monocytes and T cells and inhibits proliferation and induces apoptosis (reviewed by Commins et al., 2010). IL-10 is produced mainly by regulatory T lymphocytes, monocytes and B cells and inhibits production of several cytokines such as IFN-γ, IL-4, IL-5, IL-1β, IL-6, CXCL8, IL-12 and TNF-α (reviewed by Commins et al., 2010). The chemokine IL-8 is a potent chemo-attractant and activator of neutrophils and T lymphocytes, produced by a variety of cells types including monocytes,

fibroblasts, lymphocytes and epithelial and endothelial cells (Schaefer et al., 2004). In uroepithelial cells expression of IL-8 increased rapidly after infection with uropathogenic *E. coli* and declined after 24 hours suggesting that the secretion of these chemokine is more important during the acute inflammatory response than during the chronic phase (Godaly et al., 2001)

The binding of IL-8 to the receptors CXCR-1 and CXCR-2 is essential to amplify inflammation after the early phase of recruitment of neutrophils to the site of infection, in order to eliminate microorganisms (Ragnarsdóttir et al., 2011). The induction of IL-6 and IL-8 secretion, usually in concert with IL-1α and TNF-α secretion, elicits the recruitment and activation of macrophages, dendritic cells, neutrophils and T lymphocytes to the site of infection, as well as induction of B-cell responses and enhancing T-helper cells (Schaefer et al., 2004; reviewed by Tosi, 2005). Production of several soluble antagonists of IL-1 and TNF-α can modulate their effects, including IL-1 receptor antagonist, soluble TNF-α receptor, and anti-inflammatory cytokines, especially IL-10 (reviewed by Tosi, 2005). NK cells are an important source of IFN-γ which induces the expression of the chemokines CXCL10, Mig (CXCL9) and I-TAC (CXCL11) from tissue cells (reviwed by Luster, 2002). IFN-γ is together with LPS responsible for stimulation of neutrophils and endothelium cells for further production of CXCL10 (Tamassia et al., 2007). CXCL10 is directly induced by bacterial products and virus, playing an early role in the recruitment of T cells and NK cells to the tissue (reviwed by Luster, 2002).

In cattle, during the first week postpartum, endometrial epithelium present a prominent expression of TLR4, IL-1α, IL-1β, IL-6, TNFα and IL-10 protein which may reflect that the epithelium is the first line of defense against pathogens (Herath et al., 2009b). *Ex vivo* organ cultures of bovine endometrium, and in vitro cultures of bovine endometrial epithelial and stromal cells, and peripheral blood mononuclear cells (PBMCs), all mounted inflammatory responses to *E. coli* or LPS with secretion of inflammatory mediators IL1-β, IL-6 and IL-8, and increased expression of mRNA encoding IL-1β, IL-6, IL-8 and CCL5 (Saut et al., 2014).

Recently, the inflammatory response in the infected uterine tissue during pyometra has been more closely explored. In one study of the molecular patterns involved in the uterus in pyometra, numerous (>800) genes were found upregulated (Hagman et al., 2009). Many of these genes are associated with chemokines, cytokines, inflammatory cell

extravasation, anti-bacterial action, the complement system and innate immune responses. Some of the cytokines genes that were more up-regulated were the ones encoding IL-8, IL-1, IL-6, IL-1 receptor, IL-33 and IL-18, however, no significant up-regulation of TNF-α gene transcription or of any of the interferon family members were observed (Hagman et al., 2009). In contrast, in another study, TNF-α gene transcription was up-regulated in canine pyometra endometrium compared with health endometria (Bartoskova et al., 2012). Although, different expression patterns of cytokines in serum were observed in bitches with or without pyometra-associated SIRS only C-reactive protein, IL-7, IL-8, IL-15 and IL-18 were significantly higher in bitches with SIRS (Karlsson et al., 2012).

# CHAPTER III - OESTROUS CYCLE-RELATED CHANGES IN PRODUCTION OF TOLL-LIKE RECEPTORS AND PROSTAGLANDINS IN THE CANINE ENDOMETRIUM

Silva, E.[1], Henriques, S. [1], Brito, S., Ferreira-Dias, G., Lopes-da-Costa, L., Mateus, L. (2012). *Journal of Reproductive Immunology*, 96 (1-2): 45-57. doi:10.1016/j.jri.2012.07.003.

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Contribution of Sofia Henriques to this article:
Sofia Henriques participated in the laboratory work and data analysis process.

## 1. Abstract

The objectives of this study were to evaluate the following events in the canine endometrium over the course of the oestrous cycle: (i) the transcriptional profiles of genes encoding the Toll-like receptors (TLR1–TLR7 and TLR9); (ii) the transcription and protein expression levels of TLR2 and TLR4; (iii) the gene transcription profile of prostaglandin synthesis enzymes (PTGS2, PGES and PGFS); (iv) the response pattern of $PGF_2$ and $PGE_2$ following exposure of endometrial explants to LPS and LTA. TLR1–TLR7 and TLR9 genes were transcribed in the endometrium of bitches throughout the oestrous cycle, which indicates that TLR-mediated immune surveillance is an important component of the defense mechanisms within the uterus. Canine endometrial mRNA and protein expression of TLR2 and TLR4 was up-regulated at the late dioestrus and anoestrus and was the lowest in the follicular phase and early dioestrus. The decreased mRNA and protein levels observed at early dioestrus may favour implantation, but may also be linked to the high prevalence of pyometra at this stage of the oestrous cycle. After LPS and LTA stimulation, endometrial explants produced more $PGF_2$ than $PGE_2$, which may be related to the early demise of the corpus luteum observed in canine pyometra cases. Overall, these results indicate that TLRs are involved in the activation of the inflammatory response associated with pyometra in the bitch. TLRs may therefore be therapeutic targets for the control of uterine bacterial infections in the bitch and potentially in other species.

**Keywords:** TLR, prostaglandins, LPS, LTA, endometrium, bitch

## 2. Introduction

Uterine response to infection includes innate and acquired immune defense mechanisms that are regulated by the ovarian steroids, oestradiol and progesterone (Bouman, Jan Heineman, & Faas, 2005; Kida et al., 2006; G. S. Lewis, 2003; Sugiura et al., 2004). The innate mechanisms rely on germ-line-encoded TLRs that recognize and interact with conserved PAMPs synthesized by microorganisms and, thereby, initiate a cascade of signaling events that includes an early inflammatory response (Horne, Stock, & King, 2008). Of the 13 described mammalian TLRs, TLR2 and TLR4 are those best characterized with respect to innate responses to bacteria. In association with accessory molecules MD-2 and CD14, TLR4 is the signal transduction receptor for Gram-negative bacteria lipopolysaccharide (LPS) and heat shock proteins (Pioli et al., 2004). In contrast, a broader range of microbial products activates the immune response through engagement of TLR2, including LTA from Gram-positive bacteria and bacterial lipoproteins/lipopeptides from Gram-negative and Gram-positive bacteria (Zähringer et al., 2008). Recognition of some microbial products by TLR2 also appears to be dependent upon the formation of heterodimers with either TLR1 or TLR6. TLR3 binds to viral dsRNA, TLR5 binds to bacterial flagelin, TLR8 recognises ssRNA and TLR9 binds to bacterial CpG DNA (reviewed by Abrahams et al., 2005) After a pro-inflammatory stimulus such as LPS, IL-1 and/or TNF, expression of gene encoding PGES is upregulated, and coupled with prostaglandin-endoperoxide synthase 2 (PTGS2) expression, this promotes delayed $PGE_2$ synthesis (Helliwell, Adams, & Mitchell, 2004; Park, Pillinger, & Abramson, 2006). In the bitch, CEH–pyometra complex – develops in 25% of all intact female dogs. If left untreated it is lethal and patients may develop endotoxemia, sepsis or septic shock, ovariohysterectomy being the most effectivetreatment (Hagman et al., 2009). Although *E. coli* is the bacterium most commonly isolated in cases of pyometra, other bacteria that may be isolated include *Streptococcus* spp., *Klebsiella* spp., *Staphylococcus aureus*, *Pasteurella* spp., *Proteus* spp. and *Pseudomonas* spp. (Fransson et al., 1997). *E. coli* pyometra develops in the first half of dioestrus under high endogenous progesterone concentrations (Tsumagari et al., 2005). This fact was related to an impairment of the antimicrobial defenses (Kida et al.,

2006) and to an increase in endometrial *E. coli* adherence (Ishiguro et al., 2007). However, the involvement of the cascade events leading to the uterine inflammatory response is poorly understood. Recent studies indicated that the uterine inflammatory response towards *E. coli* is associated with an endometrial up-regulation of TLR2 and TLR4 mRNA (Hagman et al., 2009; Silva et al., 2010) and TLR4 expression (Chotimanukul & Sirivaidyapong, 2011). Here, we tested the main hypothesis that changes in TLR2 and TLR4 transcription and expression during the oestrous cycle may be associated with predisposition to the development of pyometra in the bitch. Information relating to cycle changes in TLR expression to development of uterine infection may also be useful to other species. In fact, transcription and expression of TRL1-10 changed along the menstrual cycle of women (Aflatoonian et al., 2007) and, transcription of these TLRs was present in the endometrium of dioestrous cows (Davies et al., 2008). As it is known that the inflammatory response can be modulated by ovarian steroids, a relationship between the hormonal environment and TLR expression is expected to be present and will be evaluated. However, the effect of the oestrous cycle phase on the endometrial response to LPS and LTA was not reported in the bitch. This will be evaluated using endometrial explants, providing novel information regarding the development of the inflammatory response to Gram-negative endotoxin-releasing bacteria and Gram- positive bacteria in different hormonal scenarios, in the bitch. The specific objectives of this study were to evaluate the following endometrial events throughout the canine oestrous cycle: (i) the transcriptional profile of TLRs 1–7 and 9; (ii) the mRNA and protein expression levels of TLR2 and TLR4; (iii) the gene transcription profile of prostaglandin (PG) synthesis enzymes (PTGS2, PGES and PGFS); (iv) the endometrial response pattern of $PGF_2$ and $PGE_2$, following the exposure to LPS and LTA.

## 3. Materials and methods

### 3.1. Animals and sample collection

Sixty-three non-pregnant and clinically normal bitches of mixed breeds, aged 1–11 years (average 3 years) were subjected to elective ovariohysterectomy (OVX), for contraceptive purposes, as requested by the owners, at the Teaching Hospital of the Faculty of

Veterinary Medicine of Lisbon and at the Portuguese League of Animal Rights. The phase of the oestrous cycle was determined based on vaginal cytology, histological observation of the ovaries and the uterus and, plasma progesterone (P4) concentrations (Table 1).

**Table 1 - Parameters used for oestrous cycle phase determination**

| Oestrous cycle phase **(n)** | Uterine morphology | Ovarian structures | Vaginal cytology | Progesterone concentrations (ng $L^{-1}$) mean±SD (range) |
|---|---|---|---|---|
| Proestrus (13) | proliferative stage | growing follicles | < 80% queratinized epithelial cells | 0.31±0.15 (0.11-0.57) |
| Oestrus (6) | proliferative stage | mature foliculles or growing corpora lutea | > 80% queratinized epithelial cells | 5.73±5.40 (1.41-13.39) |
| Early diestrus (0-30 days) (11) | proliferative or secretory stage | growing or fully developed corpora lutea | grouped intermediate and parabasal cells, metestrum and foam cells, PMN | 40.05±29.65 (10.40-71.10) |
| Mid diestrus (31-50 days) (6) | secretory stage | regressing corpora lutea | intermediate and parabasal cells | 17.88±10.57 (8.27-33.2) |
| Late diestrus (16) | secretory stage | regressing corpora lutea | intermediate and parabasal cells | 2.11±2.01 (0.52-7.10) |
| Anestrus (11) | quiescent stage | corpora albicans | small intermediated and basal cells | 0.36±0.09 (0.25-0.53) |

Only uteri without histological evidence of CEH were allocated to the study. Progesterone was assayed in duplicate samples and quantified by a validated solid-phase radioimmunoassay, without extraction, using a commercial kit (Coat-A-Count, Diagnostic Product Corporation, Los Angeles, CA, USA). The intra-assay coefficient of variation for all samples was 4.3%. Immediately after OVX, an intra-uterine swab was processed for bacteriological analysis and strips of endometrium were collected from the middle region of both uterine horns, rinsed with sterile RNase-free cold saline solution, immersed for 24 h in RNA later (Qiagen, GmbH, Hilden, Germany) and then stored at −80ºC until processed (n = 45) or used for explant culture (n = 26). For immunohistochemistry (IHC) and histological

classification, cranial and caudal uterine tissue was individually fixed for 24h in 4% neutral phosphate buffered formalin. *E. coli* pyometra uteri, described in a recent study (Silva et al., 2010), were also used for immunolocalization of TLR2, TLR4 and PTGS2 in the canine endometrium.

### 3.2. Explant processing and stimulation

To evaluate the endometrial response following the exposure to LPS and LTA, endometrial explants of different phases of the oestrous cycle were used (n = 26; follicular phase, n = 9; early dioestrus, n = 8; late dioestrus, n = 6; anoestrus, n = 3). Endometrial strips were cut into 1–3-mm pieces and thoroughly washed in Hanks' Balanced Salt Solution (HBSS) (GIBCO® , Invitrogen Corporation, New York, USA) supplemented with 0.1% bovine serum albumin (BSA) (Sigma, St. Louis, MS, USA), 100IU/mL penicillin plus 100µg/mL streptomycin (GIBCO®, Invitrogen), and 2.5µg/mL of amphotericin (GIBCO®, Invitrogen). The explants (around 100mg) were pre-incubated, before stimulation, in a six-well plate for 1h with 3mL/well of Dulbecco's Modified Eagle Medium (DMEM)/F12 (GIBCO®, Invitrogen) supplemented as above. Stimulation consisted of adding 1µg/mL of LPS or 1µg/mL of LTA, for 24h, to the culture medium. Medium alone and medium with explants served as negative controls. All incubations were performed with gentle shaking at 37ºC, in a 5% CO2 in air atmosphere (Heracell, Heraeus Instruments GmbH,Germany). After culture, supernatants were frozen in liquid nitrogen and stored at −80ºC until PG determination.

### 3.3. Reverse transcription polymerase chain reaction and quantitative real time RT-PCR

#### 3.3.1. Endometrial RNA extraction and cDNA synthesis

Uterine samples (20–30mg) were pulverized with a sterile mortar and pestle. Total RNA was extracted using the Rneasy Mini kit (Qiagen GmbH, Hilden, Germany) and DNA digestion was performed with the RNase-free DNase Set (Promega, Madison, WI, USA). RNA concentration and purity was determined in a NanoDrop 2000/2000c

spectrophotometer (Thermo Fisher Scientific). Single-stranded complementary DNA (cDNA) synthesis was performed by reverse transcription of 500ng of total RNA using the SuperScript III First-Strand synthesis SuperMix for quantitative reverse transcription polymerase chain reaction (qRT-PCR) according to the manufacturer's instructions (Invitrogen, Austin, TX, USA).

### 3.3.2. mRNA transcription of toll-like receptor genes and prostaglandin synthesis genes

The transcription of TLRs 1–7 and TLR 9 was evaluated by reverse transcription polymerase chain reaction (RT- PCR) using the prepared complementary DNA (cDNA) (Section 3.3.1) (n=15 uteri) and the TLR primer pairs described in Table 2.

PCR reactions were carried out in a 25µL reaction mixture containing 25pmol of each primer, 0.1mM of each deoxynucleotide triphosphate (Promega), 1× PCR buffer, 2mM MgCl2, 1.5–2.5µL of CDNA and 2.5U of GoTaq DNA polymerase (Promega). All runs included a negative control without cDNA and an RT-negative control (omitting reverse transcriptase). Except for the annealing temperatures, the thermal cycling conditions were identical for all primer pairs: 3min at 95ºC followed by 35 cycles of denaturation (95ºC for 1min), annealing (1 min) and extension (72ºC for 3min) and a final step at 72ºC for 5min. The annealing temperature was set between 54ºC and 56.5ºC. Amplification products were separated by electrophoresis through a 2.5% (TLR1-5), 1.5% (TLR7, TLR9) or 1% (TLR6) agarose gel, stained with ethidium bromide and the bands were visualized with the ImageMaster1 VDS System (Amersham Pharmacia Biotech).The identity of the PCR products was initially confirmed by DNA sequencing and thereafter the result was considered to be positive if the amplification product had the expected molecular size.

For quantification of TLR2 and TLR4 transcripts (n=25 uteri), the quantitative real time RT-PCR (qRT-PCR) standard curve method was employed. The standard curves were constructed for each gene, according to a method described before (Applied Biosystems, 2003; Silva et al., 2008). The quantification of PTGS2, PGES and PGFS mRNA was performed by real-time PCR relative quantification (n=19 uteri), according to a method already published (Silva et al., 2009b).

**Table 2 - Primer sequences for mRNA of target genes**

| | Target gene | Sequence (5´-3`) | Product size (bp) | GeneBank accession number |
|---|---|---|---|---|
| RT-PCR primers | TLR1 | FW - CAACAATTGGATATTAGCCAGAA<br>RV - GATTGGTTTAGGAATGCTCCTT | 192 | NM_001146143.1 |
| | TLR2 | FW - TCACTTGGGGAAACACCTCT<br>RV - TCATACGGAGGGCCAGATAG | 211 | NM_001005264.2 |
| | TLR3 | FW - GCGTGAATTTGACTGAACTCC<br>RV - TCAAGTTCTTCACGCCTCAG | 211 | XM_540020.2 |
| | TLR4 | FW - CAGCATTCCAGTTTGAAGCA<br>RV - GGAGTTGTCCGGAAAGGAAT | 174 | NM_001002950.1 |
| | TLR5 | FW - CTTCGTCTTCTCCCTGAACG<br>RV - CTGAACGTCTGGTCCTGGAT | 249 | XM_545722.2 |
| | TLR6 | FW - CAAGTTCAACCAGGATTTGG<br>RV - CCAAGCATTTCCAAGTTGTT | 512 | EU551147.1 |
| | TLR7 | FW - TGGGCTCAAGTCCTTTCACT<br>RV - CCACCAGACAAACCACACAG | 337 | NM_001048124.1 |
| | TLR9 | FW - TGAGCTCCAAGCATCCTTTC<br>RV - GATGCGGTTGGAGTACAAGG | 303 | NM_001002998.1 |
| Quantitative PCR primers | TLR2 | FW - CACTTCAATCCCCCGTTCAA<br>RW - AATAATCCACTTGCCGGGAATA | 66 | NM_001005264 |
| | TLR4 | FW - CCTCTTGTCATTGGATACACTAGCTT<br>RW - TGCTGTTGTCCTTGTTCCTTGA | 105 | NM_001002950 |
| | PGES | FW - CAGAGCCCACCGGAATGA<br>RV - GGAAGAAGACGAGGAAGTGCAT | 116 | NM_001122854 |
| | PGFS | FW - GGCCAAGAGCTTCAACGAGA<br>RV - AGGCTGCTCAGAGTCTCCATG | 93 | NM_001012344 |
| | PTGS2 | FW - GTATGAGCACAGGATTTGACCAGTA<br>RV - AATTCCGGTGTTGAGCAGTTTT | 82 | NM_001003354 |
| | OAZ1 | FW - CAGGTGGGCGAGGGAATAG<br>RW - ATGTGTTTGGAGCCCGTGA | 151 | NM_001127234 |

The real-time PCR primers, described in Table 2, were first chosen with Primer3 Software and confirmed with Primer Express® Software (Applied Biosystems, Foster City, CA, USA). The mRNA transcription of the ornithine decarboxylase antizyme 1 (OAZ1) gene was constant in our samples and, therefore, this gene was chosen as the house-keeping gene. All real-time PCR reactions were carried out in 96-well optical reaction plates (Applied Biosystems, Warrington, UK, ref. 4306737) with 12.5µL of PowerSYBR® Green PCR Master Mix (Applied Biosystems, ref. 4367659), 1µL of 1:5 diluted cDNA, 80nM of each

primer in a total reaction volume of 25µL. After analyzing the melting curves, the PCR products were run through a 2.5% agarose gel to confirm specificity. In each real-time PCR run, the lack of genomic DNA contamination in the RNA preparations was confirmed by the inclusion of reverse transcriptase negative controls.

### 3.4. Prostaglandin measurement

$PGE_2$ and $PGF_2$ concentrations in the culture medium were determined using an enzyme immunoassay kit for $PGF_2$ (Assay Designs, Inc., Ann Arbor, MI, USA) and $PGE_2$ (R&D Systems Europe, Ltd., Abingdon, UK). The intra-assay coefficients of variation were 6.7% and 7.2% for $PGE_2$ and $PGF_2$ respectively. All values were normalized to milligrams of tissue and are expressed as pg/mg tissue.

### 3.5. Immunohistochemical staining of TLR2, TLR4 and PTGS2

Owing to the restricted availability of canine specific and/or cross-reacting antibodies, immunohistochemistry analysis was limited to PTGS2, TLR4 and TLR2. Immunohistochemistry detection of PTGS2 was carried out as described previously (Silva et al., 2010). For TLR2 and TLR4 immunostaining, endogenous peroxidase was quenched by incubating the slides in 3% hydrogen peroxide in water for 30min. Antigen retrieval was performed using microwave irradiation for 3× 5 min in sodium citrate buffer (1mM, pH 6.0) and the sections were allowed to cool for 20min. Slides were then rinsed in phosphate-buffered saline (PBS) with 0.3% triton (for TLR4) or only PBS (for TLR2) and incubated with blocking solution (PBS with 0.1% Tween + 5% goat serum + 2.5% BSA for TLR4 and PBS + 5% mouse serum + 2.5% BSA for TLR2) for 1h at room temperature. Thereafter, slides were incubated overnight at 4ºC with the primary antibody for TLR4 [1:100, rabbit polyclonal anti-human TLR4 antibody (ab13556), Abcam] or TLR2 [1:50, goat polyclonal anti-human TLR2 antibody (ab1655), Abcam]. As secondary antibodies, peroxidase conjugate polyclonal goat anti-rabbit (dilution 1:100, Dako) and peroxidase monoclonal anti-goat/sheep IgG clone GT-34 (dilution 1:100, Sigma) were used for anti-TLR4 and anti-TLR2 staining, respectively. The staining was developed by incubating the slides with the substrate

diaminobenzidine (DAB; Impact-DAB, Vector Laboratories) until brown color is detected. Staining without the primary antibody and staining with the isotype-matched irrelevant monoclonal antibody were used as negative controls. Human endometrium and dog spleen were used as positive control tissues.

The distribution of TLR2 and TLR4 immunoexpression was studied for different endometrial cell types: the surface epithelium, the superficial glandular epithelium, basal glandular epithelium and stromal cells. The intensity of staining for TLR2 and TLR4 was scored as negative (0), weak (1), moderate (2) and strong (3).

### 3.6. Statistical analysis

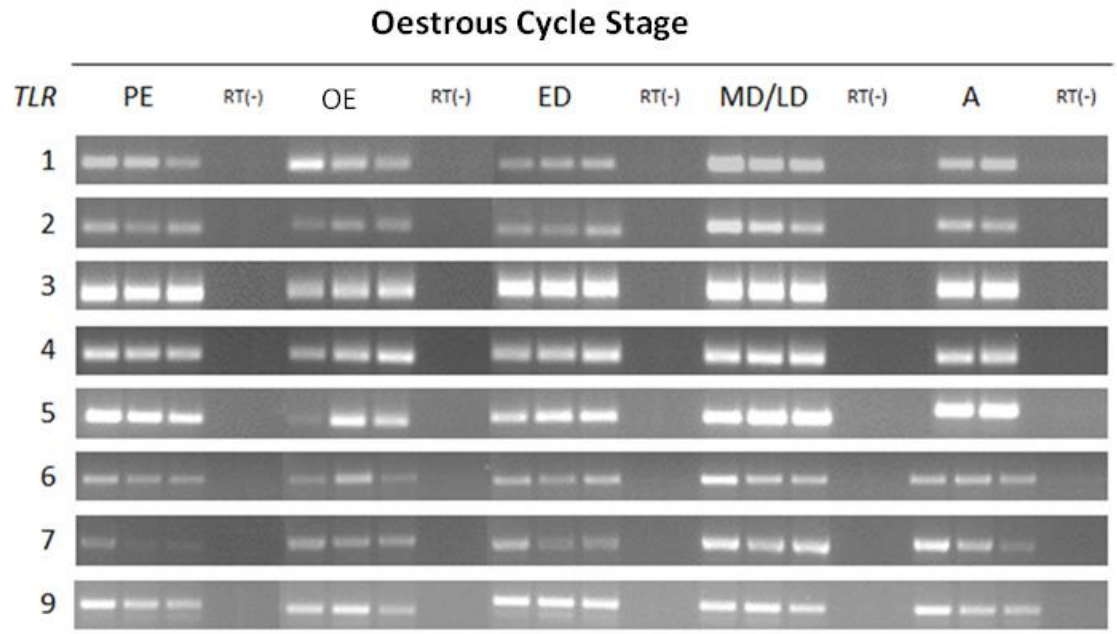
Data were analyzed through a statistical software package (Statistica 5.0, StatSoft Inc., Tulsa, OK, USA, 1995). Prostaglandin concentration data were log-transformed (log x + 1) and analyzed using the MANOVA procedures with two fixed effects: phase of the oestrous cycle (n = 4; follicular phase, including proestrus and oestrus, dioestrus, end of dioestrus, anoestrus) and type of stimulus (n = 3; control, LPS, LTA). Significant effects were further analyzed and means compared using the LSD test. All other data were analyzed using the non-parametric Mann–Whitney U test. Significance was determined at the 5% confidence level ($p < 0.05$).

## 4. Results

### 4.1. Presence of TLR1–7 and 9 gene transcripts in the canine endometrium throughout the oestrous cycle

Figure 4 shows the presence of TLR (1–7 and 9) gene transcripts in the canine endometrium throughout the oestrous cycle. As shown, gene transcripts of all TLRs were detected in all phases of the oestrous cycle.

**Figure 4 - Presence of *TLR 1-7 and 9* gene transcripts in the canine endometrium throughout the oestrous cycle**



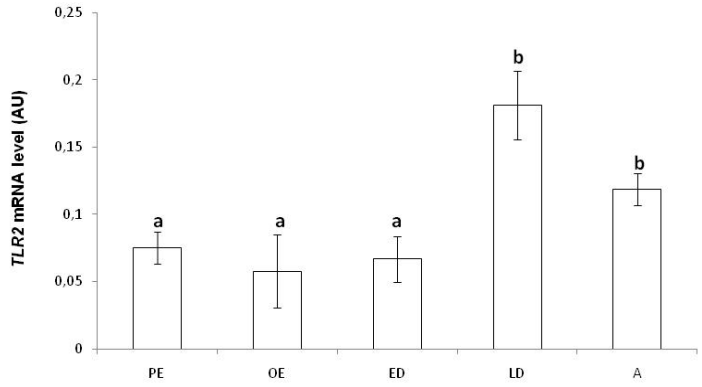

PE: proestrus (n=3); OE: oestrus (n=3); ED: early diestrus (n=3); MD/LD: mid/late diestrus (n=3); A: anestrus (n=2 for TLRs 1-5 and n=3 for TLRs 6, 7 and 8). For each TLR gene, the PCR result represents the same animal in the same order.

## 4.2. Transcription levels of TLR2 and TLR4 genes in the canine endometrium throughout the oestrous cycle

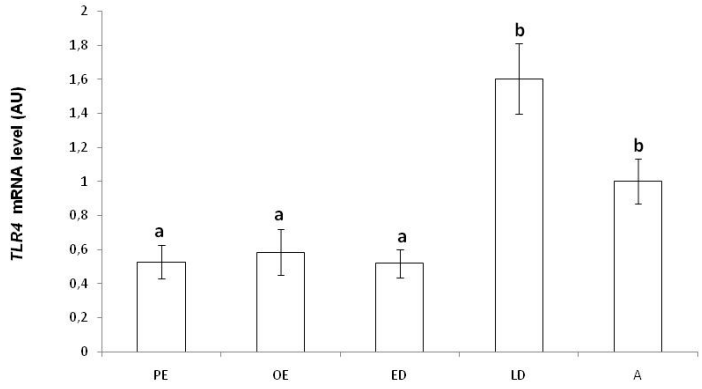
Figure 5 shows the TLR2 and TLR4 mRNA levels in the canine endometrium throughout the oestrous cycle (n = 25). As shown, TLR2 (Fig. 5A), and TLR4 (Fig. 5B) gene transcription were higher ($p < 0.05$) at the end of dioestrus and anoestrus than at the follicular phase (proestrus and oestrus) and the first half of dioestrus.

**Figure 5 - mRNA level (Arbitary Units, AU) evaluated by real-time PCR for the genes *TLR2* (A) and *TLR4* (B) in the endometrium of the bitch during the oestrous cycle (n=25)**

**A)**




**B)**



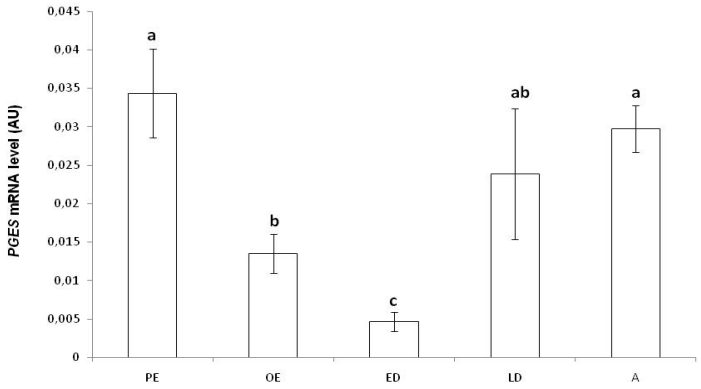

Data is given as mean±SEM. $^{ab}p < 0.05$ (Mann–Whitney U-test). PE: proestrus (n=6); OE: oestrus (n=3); ED: early diestrus (n=6); LD: late diestrus (n=6); A: anestrus (n=4).

### 4.3. Transcription levels of PGES, PGFS and PTGS2 genes throughout the oestrous cycle

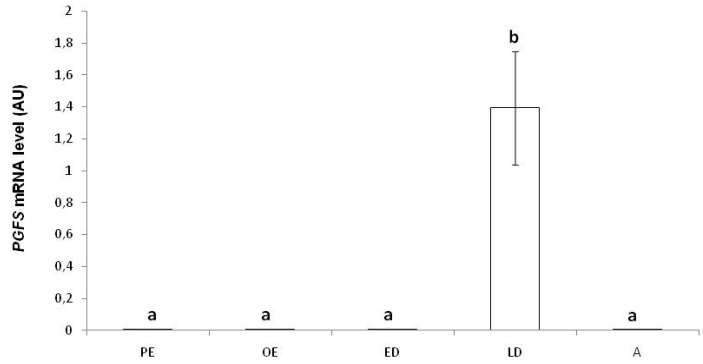
Endometria from oestrus and early dioestrus had lower mRNA transcription levels of PGES, compared with other oestrous cycle phases ($p < 0.05$; Fig. 6A). A higher transcription level of PGFS mRNA was observed in late dioestrus than at the follicular phase (proestrus and oestrus), early dioestrus and anoestrus ($p < 0.05$; Fig. 6B). PTGS2 mRNA transcription levels were higher in the endometria of late dioestrus and anoestrus than at all the other oestrous cycle phases ($p < 0.05$; Fig. 6C). Endometria from oestrus and early dioestrus had lower PTGS2 transcription levels than endometria from proestrus ($p < 0.05$; Fig. 6C).

**Figure 6 - Relative mRNA level (Arbitary Units, AU), evaluated by real-time PCR for the genes *PGES* (A) *PGFS* (B) and *PTGS2* (C), in the endometrium of the bitch during the oestrous cycle (n=19)**

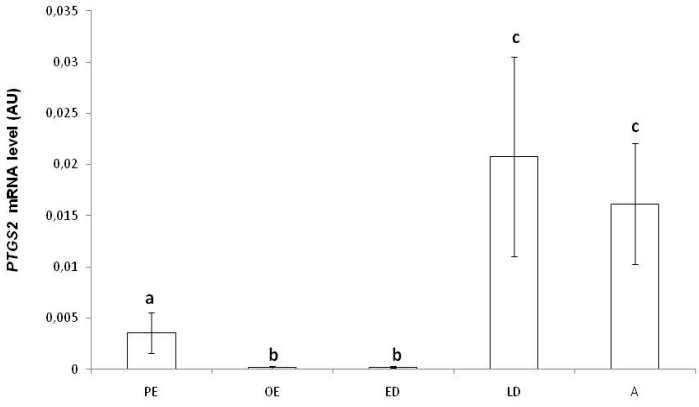
**A)**




**B)**




**C)**




Data is given as mean±SEM. Columns with different superscripts differ significantly ($p < 0.05$; Mann–Whitney U-test). PE: proestrus (n=4); OE: oestrus (n=4); ED: early diestrus (n=4); LD: late diestrus (n=4); A: anestrus (n=3).

### 4.4. Protein expression of TLR2 and TLR4 and PTGS2 in the canine endometrium throughout the oestrous cycle and in pyometra cases

Stronger positive immunostaining for TLR4 (Fig. 7) than for TLR2 (Fig. 8) was observed in endometrial surface epithelium and glandular epithelium, throughout the oestrous cycle. The expression of TLR4 was similar in basal gland epithelium throughout the oestrous cycle (Fig. 7, 2A–H; Table 3II). For the same oestrous phase, there was no difference in TLR4 immunostaining intensity between the surface epithelium and the superficial and basal glandular epithelium, except in early and mid-dioestrus, where intensity was stronger at the basal glandular epithelium (Fig. 7, 1G, H and 2E, F). A higher expression of TLR4 was observed in stromal cells during proestrus and anoestrus (Fig. 7, 3A–F). A higher number of TLR4-positive macrophages were observed at late dioestrus and anoestrus (Table 3II).

**Figure 7 - Immunostaining of TLR4 in healthy bitch endometrium during the oestrous cycle**

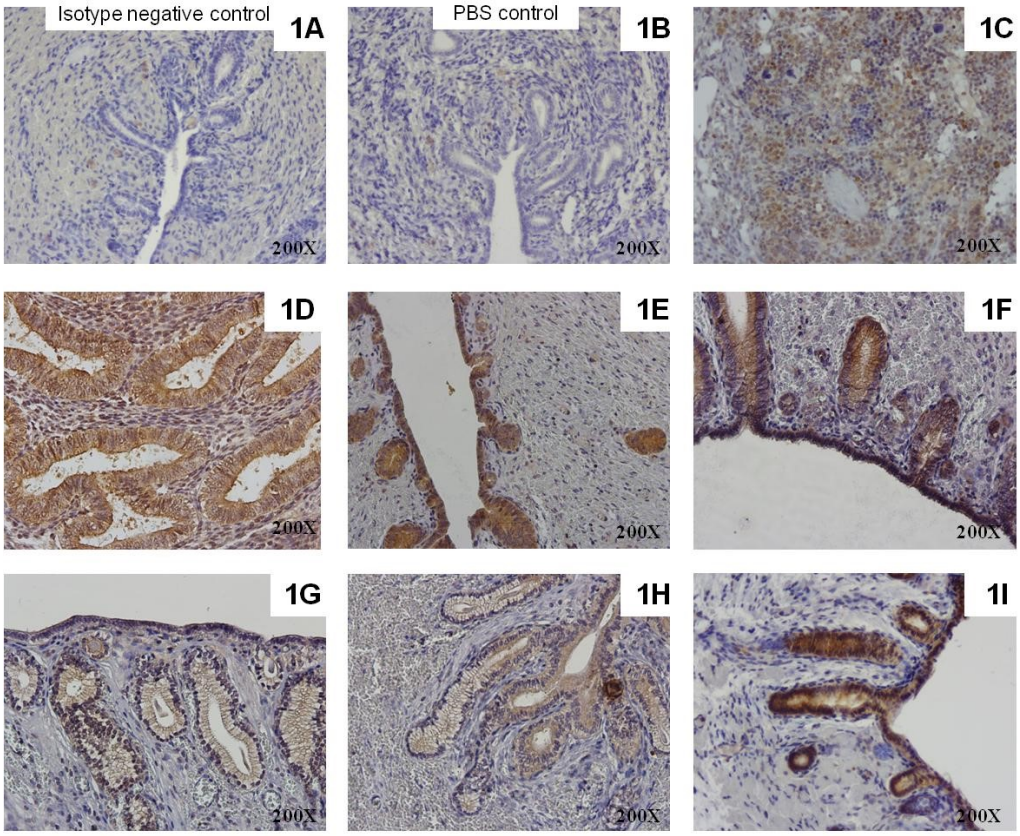
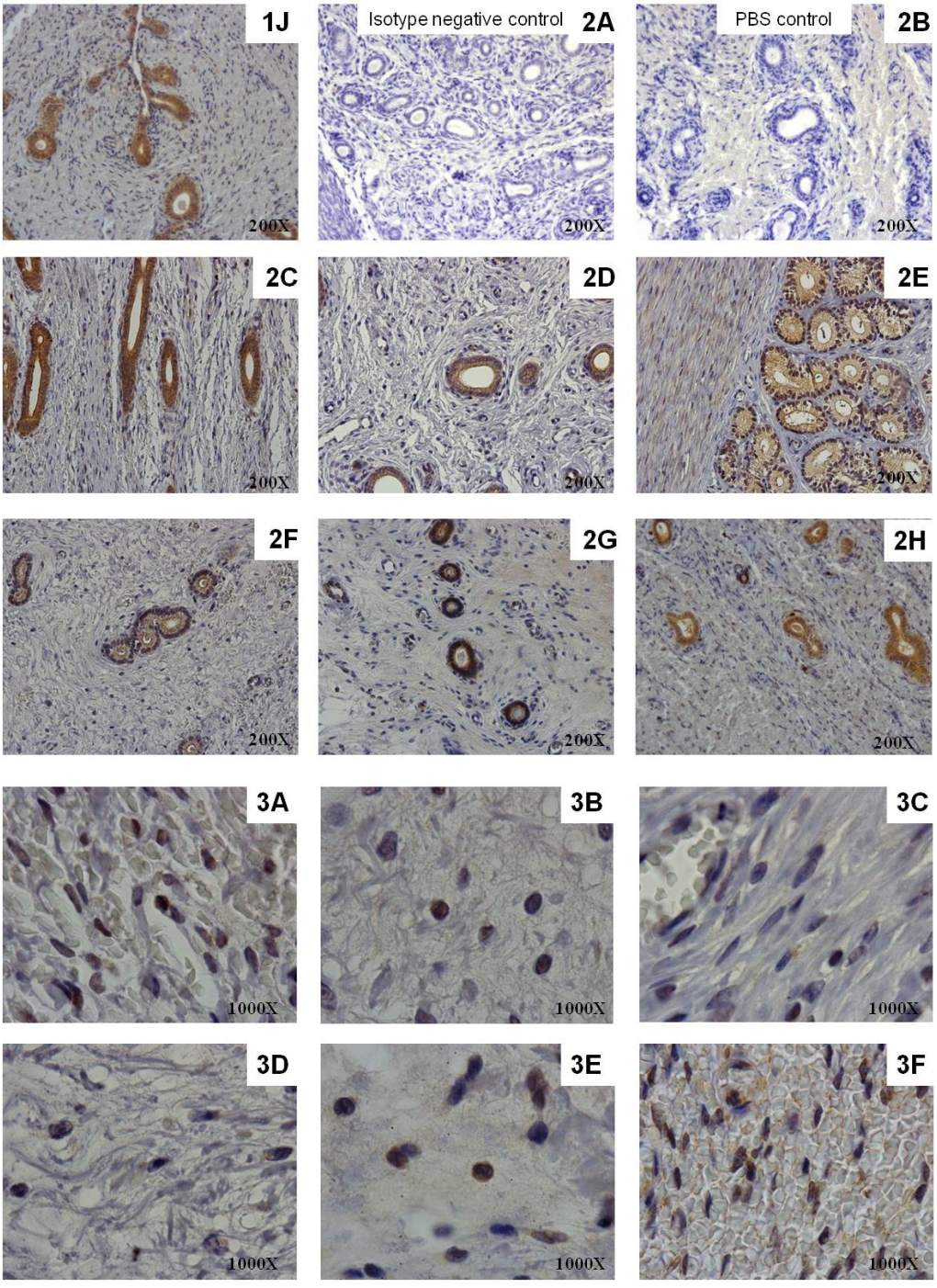


**Figure 7 (continuation) - Immunostaining of TLR4 in healthy bitch endometrium during the oestrous cycle**




TLR4 expression in surface epithelium and superficial gland epithelium (1A-1J): 1A- isotype negative control; 1B-Control sample omitting the primary antibody; 1C- Dog spleen (tissue positive control); 1D- Human endometrium (tissue positive control); 1E- Proestrus; 1F- Oestrus; 1G- Early Diestrus; 1H- Mid Diestrus and 1I- Late Diestrus and 1J- Anestrus. TLR4 staining in the surface epithelim and superficial gland epithelium was stronger at Proestrus, Late Diestrus and Anestrus and weaker at Early and Mid Diestrus. TLR4 expression in the basal glands epithelium (2A-2H): 2A- isotype negative

control; 2B-Control sample omitting the primary antibody; 2C- Proetrus; 2D- Oestrus; 2E- Early Diestrus; 2F- Mid Diestrus; 2G- Late Diestrus and 2H- Anestrus. Basal glands showed a moderate/strong staining at all oestrous cycle phases. Expression of TLR4 in stromal cells (3A-3F): 3A- Proestrus; 3B- Oestrus; 3C- Early Diestrus, 3D- Mid Diestrus; 3E- Late Diestrus and 3F- Anestrus. The number of stromal cells with positive staining for TLR4 was higher at Proestrus and Anestrus.

**Table 3 - The mean expression score for TLR2 and TLR4 in different structures of the canine endometrium throughout the estrous cycle**

**(I)**

| **TLR2 expression** | | **Proestrus** | **Oestrus** | **Early Diestrus** | **Mid Diestrus** | **Late Diestrus** | **Anestrus** |
|---|---|---|---|---|---|---|---|
| | Structures | Immunohistochemistry scoring | | | | | |
| | Surface Epithelium | 2 | 2 | 0 | 1 | 2 | 2 |
| | Superficial gland epithelium | 2 | 2 | 0 | 1 | 2 | 2 |
| | Basal gland epithelium | 2 | 2 | 0 | 0 | 2 | 2 |
| | Stroma | 2 | 1 | 0 | 1 | 2 | 2 |
| | Blood vessels | 1 | 1 | 0 | 1 | 2 | 2 |
| | Macrofages | NF | NF | NF | NF | 2 | 2 |

NF- macrophages were not observed

**(II)**

| **TLR4 expression** | | **Proestrus** | **Oestrus** | **Early Diestrus** | **Mid Diestrus** | **Late Diestrus** | **Anestrus** |
|---|---|---|---|---|---|---|---|
| | Structures | Immunohistochemistry scoring | | | | | |
| | Surface Epithelium | 3 | 2 | 1 | 1 | 2 | 3 |
| | Superficial gland epithelium | 3 | 3 | 1 | 1 | 3 | 2 |
| | Basal gland epithelium | 3 | 3 | 2 | 2 | 3 | 2 |
| | Stroma | 3 | 1 | 1 | 1 | 1 | 3 |
| | Blood vessels | 2 | 1 | 1 | 1 | 1 | 2 |
| | Macrofages | NF | NF | NF | NF | 2 | 2 |

NF- macrophages were not observed

The intensity of staining for TLR2 and 4 was scored as negative (0), weak (1), moderate (2) and strong (3)

As observed with TLR4, a lower expression of TLR2 was observed in the surface epithelium and superficial glands epithelium during early and mid-dioestrus, compared with proestrus, oestrus, late dioestrus and anoestrus (Fig. 8, 1A–J; Table 3I). The expression of TLR2 in basal gland epithelium was similar in proestrus, oestrus, late dioestrus and anoestrus (Fig. 8, 2A–H,

Table 3I) and not observed at early and mid-dioestrus. A higher number of TLR2-positive macrophages were observed at late dioestrus and anoestrus (Fig. 8, 1I and J; Table 3I).

**Figure 8 – Immunostaining of TLR2 in healthy bitch endometrium during the oestrous cycle**

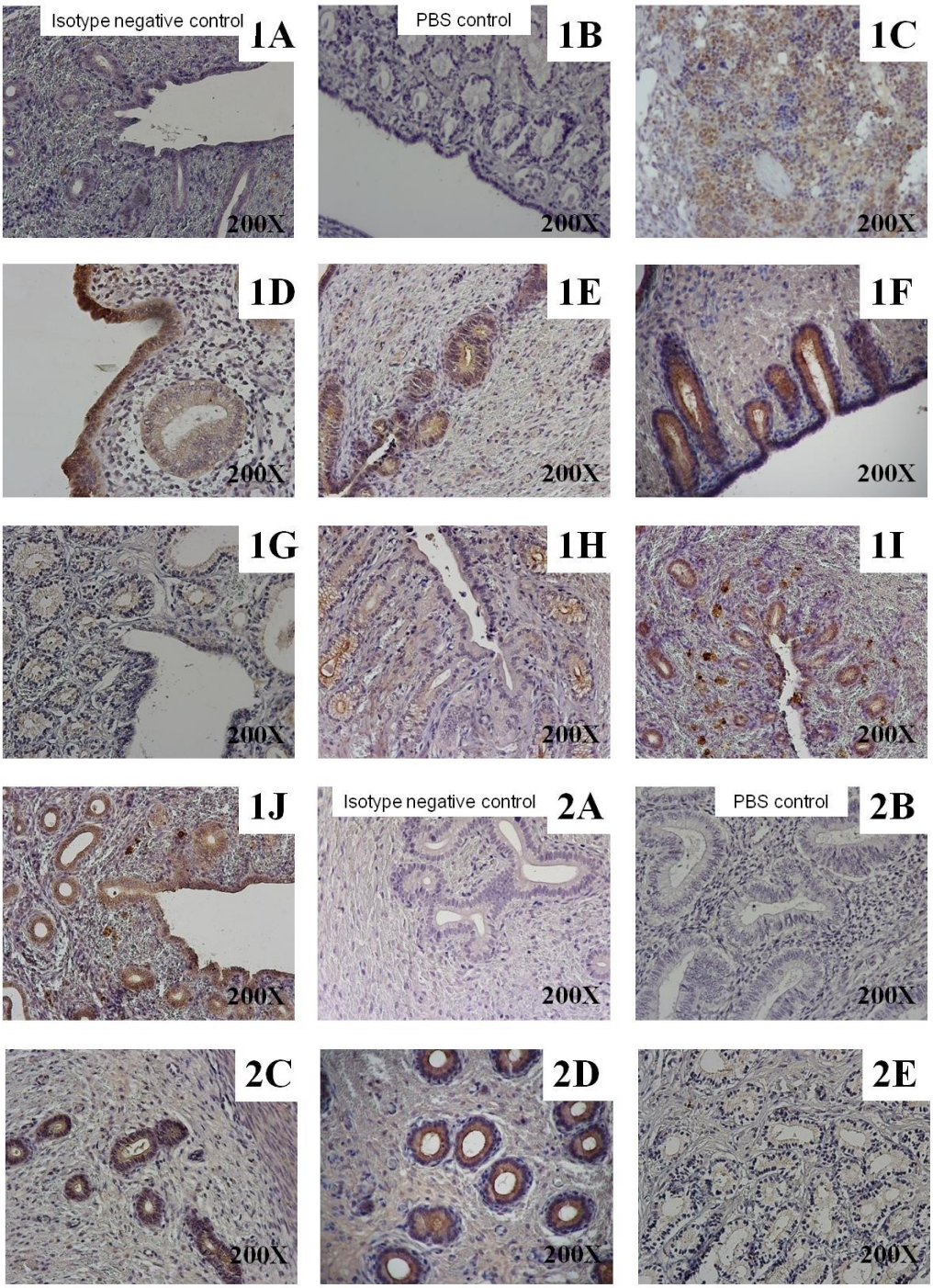
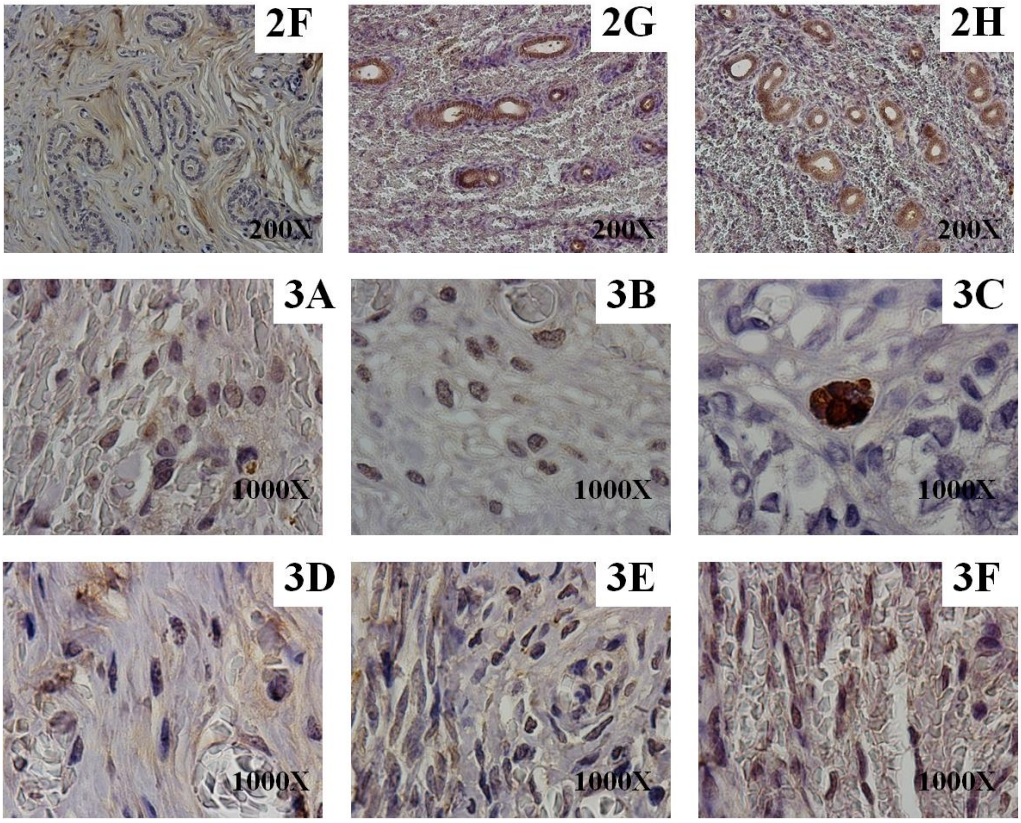


**Figure 8 (continuation) – Immunostaining of TLR2 in healthy bitch endometrium during the oestrous cycle**

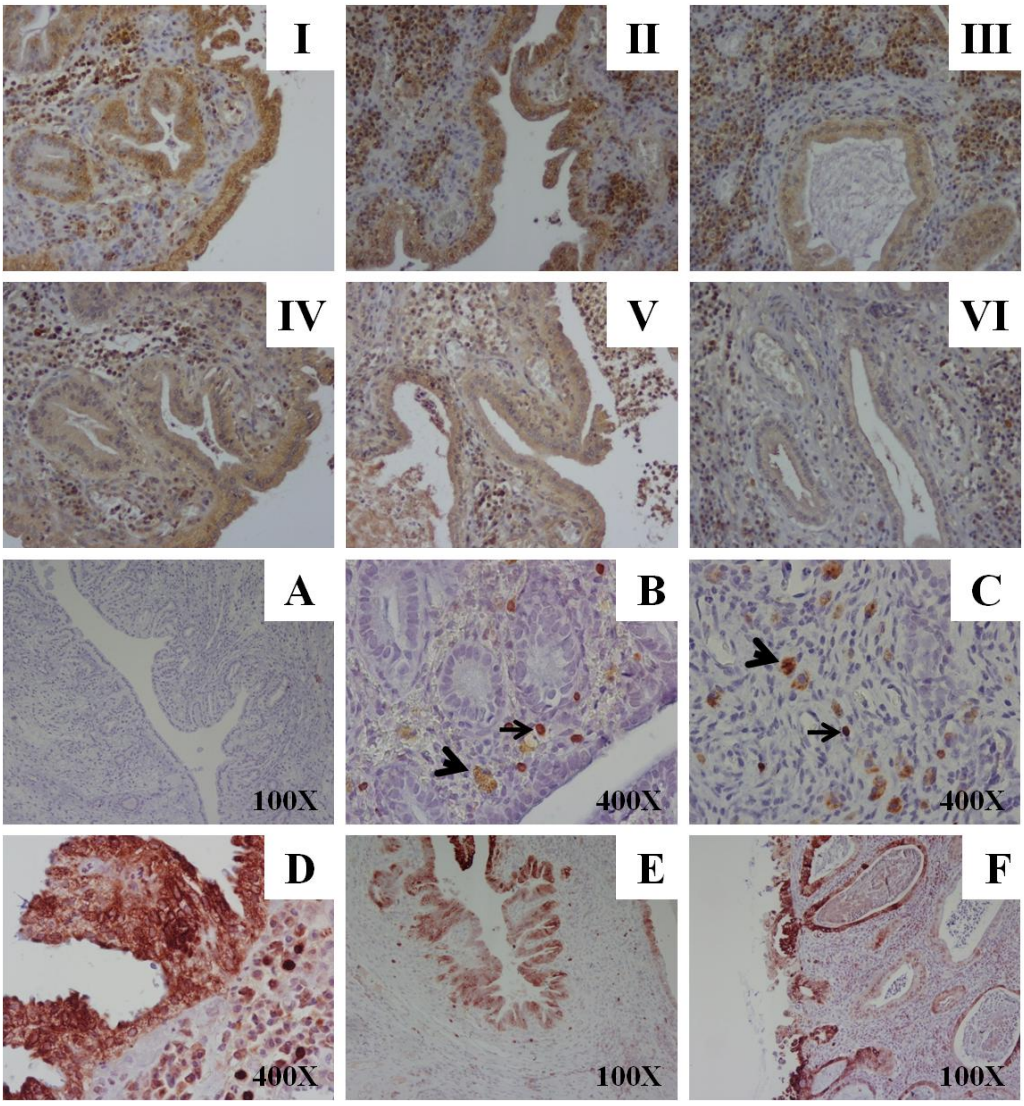



TLR2 expression in surface epithelium and superficial gland epithelium (1A-1J): 1A- isotype negative control; 1B-Control sample omitting the primary antibody; 1C-Dog spleen (tissue positive control); 1D- Human endometrium (tissue positive control); 1E- Proetrus; 1F- Oestrus; 1G- Early Diestrus; 1H- Mid Diestrus; 1I- Late Diestrus and 1J- Anestrus. TLR2 staining in the surface epithelium and superficial gland epithelium was stronger at Late Diestrus and Anestrus and weaker at Early and Mid Diestrus. TLR2 expression in the basal glands epithelium (2A-2H): 2A- isotype negative control; 2B- Control sample omitting the primary antibody; 2C- Proetrus; 2D- Oestrus; 2E- Early Diestrus; 2F- Mid Diestrus; 2G- Late Diestrus and 2H- Anestrus. Basal glands showed a moderate/strong staining at all oestrous cycle phases with the exception of Early and Mid Diestrus where there was no staining observed. Expression of TLR2 in stromal cells (3A-3F): 3A- Proestrus; 3B- Oestrus; 3C- Early Diestrus, 3D- Mid Diestrus; 3E- Late Diestrus and 3F- Anestrus. The number of stromal cells with positive staining for TLR2 was higher at Late Diestrus and Anestrus.

In pyometra uteri, the expression of TLR4 was more evident than TLR2 in the surface epithelium and superficial gland epithelium (Fig. 9I, II and IV, V). Strong expression of TLR4 and moderate/weak expression of TLR2 were observed in the glandular epithelium (Fig. 9III and VI). A marked expression of both TLRs could be observed in lymphocytes and neutrophils, which heavily infiltrate the endometrial stroma and glands (Fig. 9).

PTGS2 immunostaining was scattered and restricted to cells in the stroma (lymphocytes and macrophages) and small capillaries in normal endometria. This observation was more evident in endometria of late dioestrus and anoestrus (Fig. 9B and C). In pyometra endometria, strong staining was observed in surface epithelium and glandular epithelium as well as in the stroma because of the heavy infiltration of lymphocytes and neutrophils (Fig. 9D–F).

**Figure 9– Immunostaining of TLR4 (I-III) and TLR2 (IV-VI) and PTGS2 in canine pyometra endometrium**




The expression of TLR4 in the surface epithelium and superficial glandular epithelium (I, II) was more evident that of TLR2 (IV, V). Stronger immunostaining of TLR4 (III) was also observed in basal glandular epithelium compared to TLR2 (VI). A strong expression of both TLRs could be observed in lymphocytes and neutrophils, which heavily infiltrates the endometrial stroma and

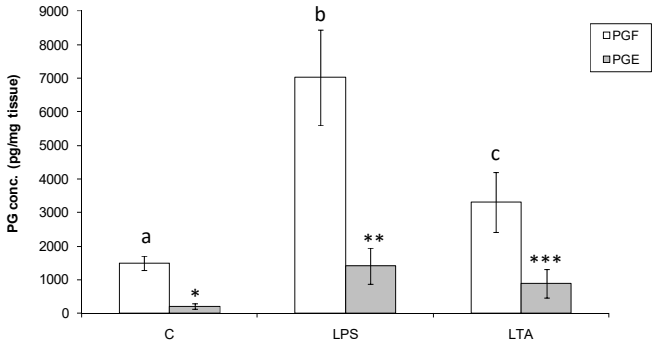
glands. A marked staining of PTGS2 was observed in surface epithelium and glandular epithelium as well as in the stroma due to the heavy infiltration of lymphocytes and neutrophils (D, E, F). Expression of PTGS2 in healthy uteri was scattered and restricted to cells in the stroma (lymphocytes, black arrow; macrophages, black arrow head) and small capillaries in normal endometria. This observation was more evident in endometria of late diestrus (B) and anestrus (C). Negative control (isotype or PBS) (A).

## 4.5. Prostaglandin production by endometrial explants after LPS or LTA stimulation

In order to determine the optimal LPS and LTA concentrations for explant stimulation of prostaglandin release, endometrial explants from non-pregnant bitches (n=4) were incubated with four concentrations of LPS (0, 0.01, 0.1, 1g/mL) and three concentrations of LTA (0.1 and 10g/mL) over 24h. Maximal concentrations of $PGF_2$ and $PGE_2$ were obtained with 1g/mL LPS ($p<0.01$) and, $PGF_2$ and $PGE_2$ concentrations were not different after explant stimulation with 1 or 10g/mL of LTA. Therefore, 1g/mL of both LPS and LTA were used for stimulation of endometrial explants (n=26).Considering all phases of the oestrous cycle together, stimulated endometrial explants secreted more $PGF_2$ (for LPS and LTA stimulation: $p <0.0001$) and $PGE_2$ (for LPS stimulation: $p <0.0001$; for LTA stimulation: $p < 0.01$) than unstimulated controls. LPS stimulation induced a higher release of both PGs compared with LTA stimulation ($p<0.05$) (Figure 10).

Table 4 shows the mean $PGF_2$ and $PGE_2$ production by endometrial explants from different oestrous cycle phases, after stimulation with LPS or LTA. As shown in this table, $PGF_2$ and $PGE_2$ production from late dioestrus endometria was higher than at the other oestrous cycle phases ($p<0.05$). Also, early dioestrus-stimulated endometria released more $PGF_2$ than endometria from the follicular phase ($p<0.01$).

**Figure 10 - Prostaglandin production (pg/mg of tissue) by endometrial explants (n=26) after 24 hours of incubation without (C) or with LPS (1µg/mL) and LTA (1µg/mL)**




Data is given as mean±SEM (error bars). $PGF_{2\alpha}$: $^{ab}p<0.0001$; $^{ac}p<0.01$; $^{bc}p<0.05$; $PGE_2$: $^{*,**}p<0.0001$; $^{*,***}p<0.0001$; $^{**,***}p<0.05$

**Table 4 - $PGF_{2\alpha}$ and $PGE_2$ production by tissue explants after LPS (1 µg/mL) or LTA (1 µg/mL) challenge**

| Phase of oestrous cycle | LPS (1 µg/mL) | | LTA (1 µg/mL) | |
|---|---|---|---|---|
| | **$PGF_{2\alpha}$** (pg/mg tissue) | **$PGE_2$** (pg/mg tissue) | **$PGF_{2\alpha}$** (pg/mg tissue) | **$PGE_2$** (pg/mg tissue) |
| Follicular (n=9) | 410.9±104.6 [a] | 431.5±74.2 [a] | 226.9±74.7 [a] | 267.1±74.5 [a] |
| Diestrus (n=8) | 1310.5±244.6 [b] | 234.9±40.5 [a] | 574.4±108.1 [b] | 195.5±34.5 [a] |
| Late diestrus (n=6) | 24058.5±9495.7 [c] | 5473.9±1734.9 [b] | 11233.0±4127.4 [c] | 3419.8±1703.0 [b] |
| Anestrus (n=3) | 568.9±237.3 [a] | 320.9±67.9 [a] | 231.6±39.6 [a] | 171.9±58.8 [a] |
| | $^{ac, bc}p< 0.001$<br>$^{ab}p< 0.01$ | $^{ab}p< 0.001$ | $^{ac, bc}p< 0.001$<br>$^{ab}p< 0.05$ | $^{ab}p< 0.05$ |

## 5. Discussion

Transcription of TLR1–7 and 9 was detected in the canine endometrium in all phases of the oestrous cycle. This observation indicates that canine endometrium is able to recognize a large variety of PAMPs and to orchestrate an innate immune response against bacterial and viral pathogens. As transcription of the TLR1–10 genes was also detected in the uterus during the human menstrual cycle (Aflatoonian et al., 2007; Schaefer et al., 2004) and the bovine dioestrus (Davies et al., 2008), activation of these genes constitutes a well-conserved pathway for the triggering of the inflammatory response within the uterus.

Differential endometrial TLR transcription and expression occurred during the oestrous cycle, indicating the regulatory role of ovarian steroids. Although the lowest transcription levels of TLR2 and TLR4 were observed during the follicular phase and the first half of dioestrus, protein expression was high only during the follicular phase. This observation might be related to the uterine contamination normally occurring during proestrus and oestrus, resulting from ascending bacteria from the vagina (Kustritz, 2006). Also, oestrogen has been shown, in mice, to promote the expression of TLR4 (Rettew, Huet, & Marriott, 2009). Contact with commensal bacteria during the follicular phase could induce up-regulation of TLR2 and 4 expressions and an inflammatory environment potentially harmful for fertility. However, it was proposed that commensal bacteria, namely Streptococcus spp., may play a protective competitive role against pathogenic bacteria (Groppetti, Pecile, Barbero, & Martino, 2012).

During the first half of dioestrus, the low transcription and protein expression of both TLRs might have two implications. The early dioestrus corresponds roughly to the implantation window of the canine embryo (16–18 days following the LH peak) (Tsutsui, 1976). Low transcription and expression of TLRs at this stage may help implantation, by reducing the inflammatory reaction against the conceptus. On the other hand, the low transcription and expression of TLR2 and TLR4 when progesterone concentrations are peaking may impair the antimicrobial defenses and could potentially be associated with the increased susceptibility to pyometra observed at this stage (Tsumagari et al., 2005).

Dissociation between protein expression and gene transcription of both TLRs was evident during late dioestrus and anoestrus. The higher transcription levels of TLR2 and TLR4

during these phases, compared with the follicular phase, might be linked to the high number of macrophages and lymphocytes present in the endometrium at those stages, due to the high apoptotic index of the stromal cells and the degeneration of the luminal epithelium (Chu, Lee, & Wright, 2006). In this regard, our results partially contradict the ones presented by Chotimanukul & Sirivaidyapong (2011) in which a high level of TLR4 expression was observed in the glandular epithelium and stroma at the dioestrus phase. However, these authors did not differentiate early from mid and late dioestrus.

The strong expression of TLRs observed in pyometra cases is in agreement with data presented by Chotimanukul & Sirivaidyapong (2011) and reflects the previous observation that TLR2 and TLR4 genes were up-regulated in the endometrium of pyometra bitches (Hagman et al., 2009; Silva et al., 2010). The activation of TLR2 and TLR4 triggers an immune response that induces an increase in PG synthesis (Helliwell et al., 2004). In pyometra cases, we have shown that prostaglandin synthesis genes were up- regulated and endometrial concentrations of $PGE_2$ and $PGF_2$, were high, which could further regulate the local inflammatory response (Silva et al., 2010). In this study we also observed that PTGS2 protein expression was up- regulated in pyometra uteri.

Prostaglandin synthesis enzyme transcripts were low in the follicular and early dioestrus phases. Kowalewski *et al*. (2010) have also shown that during pre-implantation, PTGS2, PGFS and PTGES are transcribed at a low level and in situ hybridization located PTGS2 solely in the myometrium.

The observation of high PGFS mRNA transcription during late dioestrus may indicate that the expression of PGFS is regulated at the post-transcriptional level, because low PGFM concentrations were reported in the peripheral plasma (Luz, Bertan, Binelli, & Lopes, 2006b). Alternatively, the amount of $PGF_2$ produced may not be sufficient for being detected at the peripheral level, as suggested by Luz *et al.* (2006b). As endometrial $PGF_2$ is not involved in luteal regression, its production in late dioestrus may be involved in functional and structural changes occurring in the uterus at this time (Luz, Bertan, Binelli, & Lopes, 2006a), as discussed above.

High PGES transcription was observed at late dioestrus and anoestrus. This may be related to apoptotic clearing occurring in the endometrium during these stages. In fact, in contrast to phagocytosis of pathogens, apoptotic clearing does not lead to inflammation.

Instead, anti-inflammatory cytokines such as transforming growth factor 1 and $PGE_2$ are produced (Fadok et al., 1998).

In order to simulate in vitro the endometrial exposure in vivo to Gram-positive and -negative bacteria, endometrial explants were stimulated respectively with LTA and LPS and $PGF_2$ and $PGE_2$ were measured in the culture medium. Both PGs were produced, indicating that endometrial explants responded to the stimuli, with higher PG release induced by LPS than LTA stimulation. The highest basal and stimulated $PGF_2$ and $PGE_2$ production were observed in endometrial explants of late dioestrus, which might be associated with the high transcription levels of TLR2, TLR4 and genes coding for prostaglandin synthesis enzymes.

Regardless of the oestrous cycle phase, endometrial explants produced higher concentrations of $PGF_2$ than of $PGE_2$, as also reported for pyometra cases (Silva et al., 2010). Interestingly, this high ratio of $PGF_2$:$PGE_2$ was not observed in bovine and ovine endometrial explants after stimulation with LPS (Herath et al., 2009a; Vagnoni, Abbruzzese, Christiansen, & Holyoak, 2001). In the cow, an increase in the uterine fluid $PGE_2$ concentrations during the puerperium was associated with the persistence of the uterine infection (Mateus, da Costa, Diniz, & Ziecik, 2003) and the prolonged lifespan of the corpus luteum (Herath et al., 2009a). In the bitch, around 60% of pyometra cases are associated with serum P4 concentrations lower than 1ng/l at the time of diagnosis (England, Freeman, & Russo, 2007; Fieni, 2006), while in the normal mid-dioestrus P4 concentrations are around 10–30ng/l. The early luteolysis observed in *E. coli* pyometra cases is probably the result of the high $PGF_2$ concentrations produced by the uterus. In fact, serum PGFM concentrations were higher in bitches with pyometra than in bitches with a normal or cystic hyperplasic dioestrus uterus (Hagman et al., 2006).

In conclusion, TLR genes are transcribed in the endometrium of bitches throughout the oestrous cycle, which indicates that TLR-mediated immune surveillance is an important component of the defense mechanisms within the uterus. The decreased transcription and expression levels observed at early dioestrus might be favorable to implantation, but might also be linked to the high prevalence of pyometra at this stage of the oestrous cycle. After LPS and LTA stimulation, endometrial explants produced more $PGF_2$ than $PGE_2$, which might be related to the in vivo early demise of the corpus luteum observed in canine pyometra cases. Overall, these results indicate that TLRs are involved in the

activation of the inflammatory response against pyometra in the bitch. This prompts TLRs as therapeutic targets for the control of uterine bacterial infections in the bitch and potentially in other species.

# CHAPTER IV - VIRULENCE GENOTYPES OF *ESCHERICHIA COLI* CANINE ISOLATES FROM PYOMETRA, CYSTITIS AND FECAL ORIGIN

Mateus, L., Henriques, S., Merino, C., Pomba, C., Lopes-da-Costa, Silva, E. (2013). *Veterinary Microbiology*, 166 (3-4): 590-594. doi:10.1016/j.vetmic.2013.07.018.

Contribution of Sofia Henriques to this article:

Sofia Henriques participated in the laboratory work and data analysis process.

## 1. Abstract

Pyometra is the most common diestrual uterine disease of bitches. *E. coli* is the most frequent bacterium isolated from the uterine content of pyometra uteri and it is associated with the most severe clinical signs, leading to endotoxemia and sepsis. In this study, canine *E. coli* isolates from pyometra (n=31), cystitis (n=23) and fecal (n=26) origin were compared regarding the prevalence of 23 potential virulence traits (15 VF genes and 8 PAIs), detected by PCR assays. Overall, there was a considerable overlap between pyometra, cystitis and fecal isolates regarding the phylogenetic grouping and virulence traits. Virulence traits more prevalent in pyometra than in cystitis and fecal isolates included two PAIs (PAI IV536 and PAI ICFT073) and three VF genes (sfa/focDE, fyuA and chuA). Regardless the isolates origin, the average number of virulence traits per strain was higher in B2 than in the other phylogenetic groups (A, B1 and D). The prevalence of phylogenetic group B2 was significantly higher in pyometra (94%) than in cystitis (48%) and fecal (39%) isolates. In conclusion, pyometra isolates have a high potential of virulence and a broad virulence genotype, although being similar to a subset of cystitis and fecal isolates. This leads to the suggestion that cystitis and fecal isolates may be able to induce pyometra in receptive hosts.

**Keywords:** canine pyometra, *Escherichia coli*, pathogenicity island, phylogeneic group

## 2. Introduction

*E. coli* is the bacterium most commonly isolated in cases of canine pyometra. Its presence is normally associated with highly severe systemic signs and a potentially life-threatening situation. Pyometra *E. coli* isolates derive from the host's fecal and perineal flora (Hagman & Kühn, 2002) and are characterized by the presence of several virulence genes normally found in UPEC (Chen et al., 2003; Siqueira et al., 2009). VF genes of UPEC are usually present on PAIs. PAIs have been spread among bacteria by horizontal transfer and provide a virulence advantage allowing their adaptation to niches incapable to be colonized by commensal *E. coli* strains (Lloyd et al., 2009). UPEC PAIs were detected in *E. coli* strains from extraintestinal infections (Bingen-Bidois et al., 2002) but also in commensal isolates from fecal origin (Sabaté et al., 2006). Despite the recognized similarities in virulence genotype between *E. coli* isolates from canine pyometra and UTIs, the identification and determination of the prevalence of UPEC PAIs in pyometra *E. coli* was not reported. Here, we hypothesized that pyometra and UTIs *E. coli* share the same PAIs, although with a different prevalence, which could explain their ability to colonize and infect either the uterus or the urinary tract.

## 3. Materials and methods

### 3.1. *E. coli* strains

A total of 80 *E. coli* strains were isolated from female dogs including 31 uterine pyometra isolates (Silva et al., 2010), 23 clinical cystitis isolates (Féria, Machado, Correia, Gonçalves, & Gaastra, 2001) and 26 fecal isolates obtained from healthy bitches with no clinical history of pyometra or UTI. *E. coli* CFT073, *E. coli* 536 (both kindly provided by A. Andreu, Microbiology Department, Hospital Vall d'Hebron, Barcelona, Spain), *E. coli* J96, *E. coli* KS52 and *E. coli* ATCC 25922 were used as positive controls of the PCR reactions. The hemolytic activity, associated with the expression of *E. coli* hlyA, was evaluated on Columbia 5% sheep blood agar (bioMerieux, Marcy L'Etoile, France).

### 3.2. Detection of PAI markers, virulence factors genes and phylogenetic groups

Total *E. coli* DNA was extracted from pure cultures by a rapid boiling procedure (Féria et al., 2001). The presence of eight UPEC-PAIs was screened by multiplex PCR assays, according to Sabaté *et al*. (2006) and Bronowski *et al*. (2008). PCR reactions were split in three separate multiplex assays: multiplex A for PAIs III536, IV536 and IICFT073; multiplex B1 for PAIs IIJ96 and I536; and multiplex B2 for PAIs II536, IJ96 and ICFT073. *E. coli* isolates were also tested for the presence of fifteen VF genes as described elsewhere (Johnson & Stell, 2000b; Mellata, Ameiss, Mo, & Curtiss, 2010; Nakano, 2001; S. Schubert, Rakin, Karch, Carniel, & Heesemann, 1998; Yamamoto et al., 1995). The phylogenetic group was determined by multiplex PCR (Clermont et al., 2000; Doumith, Day, Hope, Wain, & Woodford, 2012).

### 3.3. Cluster analysis of virulence genotype

The Boolean data matrix (presence/absence of virulence factors and PAIs) of 22 characteristics and 80 operational taxonomic units (OTUs) was analyzed by hierarchic numerical methods using the software package BioNumerics (version 6.6, Applied Maths, Kortrijk, Belgium). Resemblance was computed with simple matching coefficient and agglomerative clustering was performed with the unweighted average linkage (UPGMA).

### 3.4.Statistical analysis

The prevalence of VF genes and PAI markers of different isolate origins (pyometra/UTI/feces) was compared by the Fisher's exact test (Statistica 5.0, StatSoft Inc., Tulsa, OK, USA, 1995). Results were considered significant if $p < 0.05$.

## 4. Results and discussion

This study first reports the compared prevalence of PAIs in canine *E. coli* isolates of pyometra, cystitis and fecal origin. Although these isolates showed an overlap in their virulence traits, the prevalence of UPEC PAIs markers was higher ($p<0.05$) in pyometra

(100%) than in cystitis (74%) and fecal (54%) isolates (Table 5). Seemingly, although phylogenetic group B2 was present in the three origins, the prevalence was higher ($p<0.05$) in pyometra (94%) than in cystitis (48%) and fecal (39%) isolates (Table 6). This is in accordance with phylogenetic grouping data reported by Zhang *et al.* (2002) in human *E. coli* cystitis and fecal isolates. However, Sabaté *et al*. (2006) reported a lower prevalence of phylogenetic B2 group in human fecal *E. coli* isolates. The high prevalence of B2 group observed in canine fecal isolates could be related with its higher ability to survive in canine intestine as observed in humans (Nowrouzian et al., 2005). The average number of PAIs per strain was affected by phylogenetic grouping (Table 6), being higher ($p < 0.05$) in group B2 (4.2; range 2–7) than in groups B1 (0.1), D (0.7) and A (0.6). PAI IV536 and PAI III536 were, respectively, the most and the least prevalent PAIs (Table 5), a finding in agreement with Sabate´ et al. (2006), which may be related to PAI stability in the genome (Middendorf et al., 2004). Only PAI IV536 and PAI ICFT073 were more prevalent ($p < 0.05$) in pyometra than in cystitis and fecal isolates (Table 5), indicating thatthese PAIs are potentially relevant for *E. coli* virulence in pyometra. PAI IV536 encodes for the yersiniabactin iron-uptake system, and all isolates carrying this PAI were also positive for the *fyuA* gene. However, pyometra *E. coli* strains harbored genes for multiple iron acquisition systems (*fyuA, iucD, iroN, fepA, sitA* and *chuA*) (Table 5), which may indicate a functional redundancy or/ and a higher competitive advantage for bacterial survival and persistence. The average number of iron acquisition related genes per isolate was similar in pyometra, cystitis and fecal isolates (4.7, 4.5 and 3.9 genes per isolate, respectively). However, the higher prevalence of *fyuA* and *chuA* genes in pyometra isolates (Table 5) may indicate a more relevant contribution of these factors to *E. coli* iron acquisition in the uterus as observed for siderophore receptors FyuA and IutA in the urinary tract (Garcia et al., 2011). PAI ICFT073 encodes for P fimbriae, α-hemolysin and aerobactin. The presence of this PAI marker was not always associated to the presence of *hlyA, papEF* and *iucD* genes, and all isolates positive only for PAI ICFT073 were nonhemolytic. This indicates that part of this island was deleted. This gene deletion may have a selective advantage in the persistence within the uterus, since the loss of hemolysin and P-fimbriae genes can attenuate the host immune response (Blum et al., 1994). As the same VF can be encoded by different PAIs, the isolates with multiple PAIs can lose one PAI and acquire new VFs with no effect on phenotype. Here, this is evident for α-

hemolysin, which is encoded by three PAIs (I536, II536 and IIJ96). Classically, PAI IIJ96 carries the genes for α-hemolysin, Prs fimbriae and cytotoxic necrotizing factor 1. Here, the presence of PAI IIJ96 was always associated to the presence of cnf1 and hlyA genes and to papEF gene in 96% of occasions (Fig. 11). This indicates that cnf1/hlyA and pap genes were physically linked within PAI IIJ96. None of the screened adhesin genes were specifically associated with pyometra isolates, confirming results reported by Siqueira *et al.* (2009). Pyometra, cystitis and fecal isolates carried several adhesin genes (average: 2.8, 2.4 and 1.8, respectively), which reinforces the idea of functional redundancy. *fimH* was the most prevalent adhesin gene in the three isolate origins as described by Chen *et al*. (2003), for pyometra and fecal isolates. The functional significance of *fimH* gene presence is controversial, as *fimH* expression is regulated by environmental conditions and mannose receptors availability (Schwan, 2011), and *fimH* gene knock-out reduced *E. coli* binding capacity to canine endometrium only when other adhesin genes were also deleted (Krekeler et al., 2013). The prevalence of sfa/focDE genes in pyometra isolates was 77% which is similar to that described by Chen *et al*. (2003) but higher than that observed by Siqueira *et al*. (2009). Using foc- and sfa-specific primers (Johnson & Stell, 2000b) we observed that the above high prevalence was due to the presence of F1C fimbriae encoding gene (focG), a sfa-related mannose resistant adhesin. Based on a 77% similarity, three major virulence clusters (A, B and C) were identified (Fig. 11). Cluster A included non-B2 phylogenetic group isolates (2 pyometra, 11 cystitis and 15 fecal) with an average of 5.1 virulence determinant genes. This cluster was divided into two subclusters based on the presence of the *fyuA* gene and the PAI IV536. Cluster B included mainly phylogenetic group B2 (92% B2 and 8% D) isolates (14 pyometra, 5 cystitis and 6 fecal isolates) with an average of 10.2 virulence determinants. Compared to cluster A, cluster B showed a higher prevalence of *sfa/focDE*, *focG*, *usp* genes and of PAIs ICFT073 and IV536 markers. Cluster C only included phylogenetic group B2 isolates (15 pyometra, 7 cystitis and 5 fecal isolates) with an average of 17 virulence traits. Cluster C isolates are potentially more virulent and distinguishable from isolates of other clusters by the presence of *hlyA*, *cnf1* genes and the markers for PAI I536, PAI II536, PAI III536 and PAI IIJ96. In conclusion, our results demonstrated that no VF genes or virulence traits could be specifically associated with *E. coli* pyometra isolates. These isolates were mainly assigned to the highly virulent phylogenetic group B2, indicating that this group has the

highest potential to colonize the canine uterus and establish infection. Also, pyometra *E. coli* isolates exhibited a high number of virulence factor genes and PAIs markers. However, this virulence profile was also found in a subset of cystitis and fecal isolates, leading to the suggestion that they may be potentially able to induce pyometra in receptive hosts.

**Table 5 - Comparison of prevalence of eight pathogenicity islands markers and 15 virulence factor genes between pyometra (n=31), cystitis (n=23) and fecal isolates (n=26)**

| | Nº (%) isolates | | |
|---|---|---|---|
| **Genes/PAIs** | **Pyometra (n=31)** | **Cystitis (n=23)** | **Fecal (n=26)** |
| $I_{536}$ | 15 (48.4) [(a)] | 7 (30.4) [(ab)] | 5 (19.2) [(b)] |
| $II_{536}$ | 14 (45.2) [(a)] | 7 (30.4) [(ab)] | 5 (19.2) [(b)] |
| $III_{536}$ | 1 (3.2) | 2 (8.7) | 2 (7.7) |
| $IV_{536}$ | 31 (100) [(a)] | 15 (65.2) [(b)] | 14 (53.8) [(b)] |
| $I_{CFT073}$ | 27 (87.1) [(a)] | 11 (47.8) [(b)] | 10 (38.5) [(b)] |
| $II_{CFT073}$ | 14 (45.2) [(a)] | 10 (43.5) [(ab)] | 5 (19.2) [(b)] |
| $I_{J96}$ | 0 (0) | 0 (0) | 0 (0) |
| $II_{J96}$ | 15 (48.4) [(a)] | 7 (30.4) [(ab)] | 5 (19.2) [(b)] |
| **Adhesins** | | | |
| *fimH* | 31 (100) | 22 (95.7) | 23 (88.5) |
| *papEF* | 15 (48.4) [(a)] | 9 (39.1) [(ab)] | 5 (19.2) [(b)] |
| *sfa/focDE* | 24 (77.4) [(a)] | 10 (43.5) [(b)] | 8 (30.8) [(b)] |
| *sfaS* | 0 (0) | 4 (17.4) | 3 (11.5) |
| *fogG* | 19 (61.3) [(a)] | 11 (47.8) [(ab)] | 9 (34.6) [(b)] |
| *afaBC* | 0 (0) | 1 (4.3) | 0 (0) |
| **Toxins** | | | |
| *hlyA* | 15 (48.4) [(a)] | 7 (30.4) [(ab)] | 5 (19.2) [(b)] |
| *cnf1* | 15 (48.4) [(a)] | 7 (30.4) [(ab)] | 5 (19.2) [(b)] |
| *usp* | 17 (54.8) [(a)] | 14 (60.9) [(a)] | 6 (23.1) [(b)] |
| **Siderophores** | | | |
| *chuA* | 29 (93.5) [(a)] | 15 (65.2) [(b)] | 17 (65.4) [(b)] |
| *fyuA* | 31 (100) [(a)] | 15 (65.2) [(b)] | 14 (53.8) [(b)] |
| *sitA* | 31 (100) | 23 (100) | 23 (88.5) |
| *fepA* | 26 (83.9) | 21 (91.3) | 24 (92.3) |
| *iroN* | 27 (87.1) [(ab)] | 23 (100) [(a)] | 20 (76.9) [(b)] |
| *iucD* | 3 (9.7) | 6 (26.1) | 6 (23.1) |

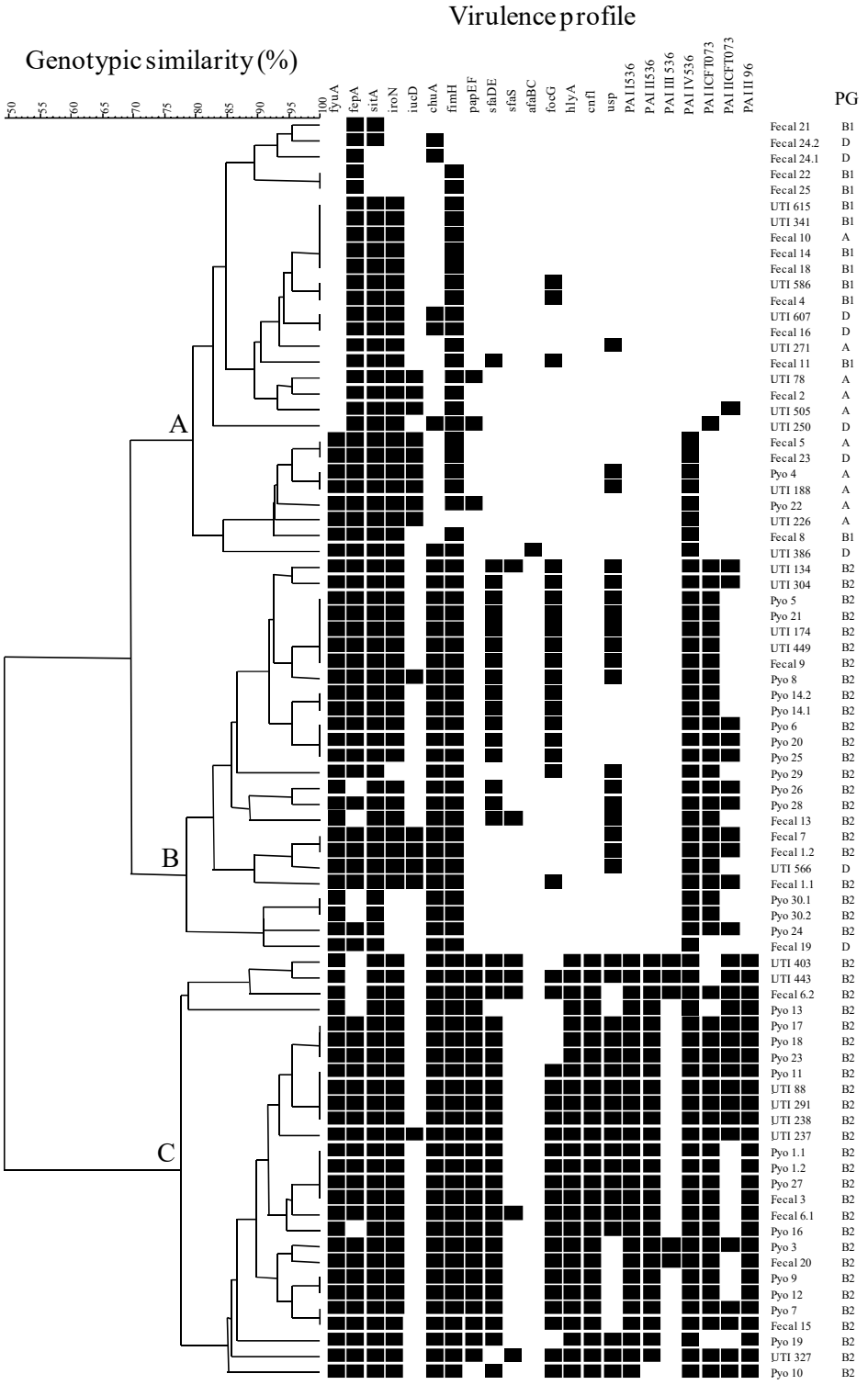
[a,b] $p < 0.05$

**Table 6 - Distribution of pathogenicity islands markers (PAI) in pyometra (n=31), cystitis (n=23) and fecal isolates (n=26), classified according to phylogenetic group.**

| Phylogenetic group | Nº isolates (%) | Nb (%) | PAIs | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | $I_{536}$ | $II_{536}$ | $III_{536}$ | $IV_{536}$ | $I_{CFT073}$ | $II_{CFT073}$ | $I_{J96}$ | $II_{J96}$ |
| **A** | | | | | | | | | | |
| **Pyometra** | 2 (6.5) | 2 (6.5) | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| **Cystitis** | 5 (21.7) | 3 (13) | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 0 |
| **Feces** | 3 (11.5) | 1 (3.8) | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| **Total** | 10 | 6 | 0 | 0 | 0 | 5 | 0 | 1 | 0 | 0 |
| **B1** | | | | | | | | | | |
| **Pyometra** | 0 (0)[c] | 0 (0) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Cystitis** | 3 (13)[cd] | 0 (0) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Feces** | 8 (30.8)[d] | 1 (3.8) | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| **Total** | 11 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| **B2** | | | | | | | | | | |
| **Pyometra** | 29 (93.5)[a] | 29 (93,5) | 15 | 14 | 1 | 29 | 27 | 14 | 0 | 15 |
| **Cystitis** | 11 (47.8)[b] | 11 (47.8) | 7 | 7 | 2 | 11 | 9 | 9 | 0 | 7 |
| **Feces** | 10 (38.5)[b] | 10 (38.5) | 5 | 5 | 2 | 10 | 10 | 5 | 0 | 5 |
| **Total** | 50 | 50 | 27 | 26 | 5 | 50 | 46 | 28 | 0 | 27 |
| **D** | | | | | | | | | | |
| **Pyometra** | 0 (0)[a] | 0 (0) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Cystitis** | 4 (17.4)[b] | 3 (13) | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 |
| **Feces** | 5 (19.2)[b] | 2 (7.7) | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| **Total** | 9 | 5 | 0 | 0 | 0 | 4 | 2 | 0 | 0 | 0 |

Nb, number of isolates with PAIs. [a,b] $p<0.05$; [c,d] $p< 0.01$

**Figure 11 - Clustering of the eighty *E. coli* isolates based on the presence or absence of the virulence factor genes and PAI markers**




Each column shows the results of a single gene. Black: gene present; white: gene absent. PG (phylogenetic groups) were not used for clustering, are only informative.

# CHAPTER V - GENOTYPIC AND PHENOTYPIC COMPARISON OF *ESCHERICHIA COLI* FROM UTERINE INFECTIONS WITH DIFFERENT OUTCOMES: CLINICAL METRITIS IN THE COW AND PYOMETRA IN THE BITCH

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Contribution of Sofia Henriques to this article:
Sofia Henriques participated in the laboratory work and data analysis process and co-produced the manuscript.

## 1. Abstract

*Escherichia coli* uterine infection originates different clinical outcomes in the canine and bovine species. Here, *E. coli* strains isolated from bovine clinical metritis and canine pyometra cases were analyzed by pulsed-field gel electrophoresis (PFGE), screened for 33 VF genes and for phylogenetic grouping. Bovine and canine *E. coli* isolates presented a low degree of genetic similarity. Canine *E. coli* strains belonged to phylogenetic group B2 and presented a high number of VF genes, whereas bovine *E. coli* strains belonged to phylogenetic groups B1 and A and had a low number of VF genes. In conclusion, *E. coli* strains isolated from cow clinical metritis had a low potential of virulence. In contrast, bitch pyometra *E. coli* isolates had a high virulence potential, which might be relevant in the pathogenesis of pyometra. These differences between canine and bovine *E. coli* isolates may partially explain the different outcomes of the uterine infection in the two species.

**Keywords:** *E. coli*, canine pyometra, bovine puerperal metritis, virulence factor genes

## 2. Introduction

*E. coli* is a common bacterium of the gastrointestinal flora of animals and humans. Ascending contamination of the uterus by *E. coli* of fecal origin occurs during the puerperium in the cow and at proestrus/estrus in the bitch (Chen et al., 2003; Mateus et al., 2002). *E. coli* is the most prevalent bacterium isolated during the first week postpartum from the uterus of cows that developed puerperal uterine infection (Bicalho et al., 2012; Mateus et al., 2002; Sheldon et al., 2009) and, of bitches with pyometra (Chen et al., 2003; Hagman & Kühn, 2002; Siqueira et al., 2009). Around 40% of lactating cows develop metritis within two weeks of calving (Sheldon et al., 2009) and about 25% of intact female dogs develop pyometra during diestrus before 10 years of age (Hagman, 2012). However, the outcomes of these diseases are very different. In the puerperal cow, *E. coli* favors the establishment of *Trueperella pyogenes* and Gram-negative anaerobes in the uterus, inducing an infection that is usually associated with abnormal postpartum ovarian function and impaired reproductive performance (Mateus et al., 2002; Sheldon et al., 2009), although the infection may resolve spontaneously (Mateus et al., 2002). In contrast, in the bitch, *E. coli* alone is responsible for 90% of pyometra cases, and if left untreated, bitches may develop endotoxemia, sepsis or septic shock (Hagman, 2012). The association between *E. coli* virulence traits and the development of puerperal or clinical metritis in the cow is controversial (Bicalho et al., 2012; Sheldon et al., 2010; Silva et al., 2009a). Canine pyometra *E. coli* isolates share some of the virulence factors (VFs) detected in UPEC responsible for cystitis in companion animals (Chen et al., 2003; Hagman & Kühn, 2002; Siqueira et al., 2009). This fact suggests that these VFs may be associated to the development of pyometra. Companion and production animals may act as reservoirs of resistant bacteria that can be transmitted to humans (or vice versa) by direct contact or indirectly, via the food chain (Bélanger et al., 2011; Marshall & Levy, 2011). Antibiotic resistance among metritis or pyometra *E. coli* isolates may reflect inappropriate use of antimicrobials by veterinarians, dairy farmers and dog breeders. Therefore, the evaluation of antimicrobial resistance patterns is useful in the establishment of appropriate therapeutic measures that avoid the emergence of resistant *E. coli* strains potentially harmful to humans and other animals. The purpose of this study was to compare the molecular and phenotypic characteristics of *E. coli* isolates recovered from

the uterus of cows with clinical metritis and of bitches with pyometra, in an attempt to correlate their virulence potential with the different clinical outcomes of the diseases. The genomic characterization included the detection of 33 VF-genes by PCR and the evaluation of the genetic similarity by PFGE. The antimicrobial resistance profile was evaluated using a MIC-based broth microdilution.

## 3. Materials and methods

### 3.1. *E. coli* isolates

Bovine isolates (n=20) were recovered from uterine swabs obtained from 15 dairy cows with clinical metritis. The clinical characterization of these animals was previously described by Silva *et al*. (2009b).In four of these animals, multiple isolates (2–3) recovered during the course of the infection were used. Isolates from the same cow were previously identified as different clonal types (Silva et al., 2009a). Canine isolates (n = 29) were recovered from uterine swabs of bitches with pyometra (n=27), after ovariohysterectomy. Pyometra was diagnosed based on case history, clinical signs, the ultrasonographic finding of an enlarged, fluid-filled uterus, and haematologic and biochemical profiles. The average age of bitches was 9.7 years (range 4–13 years). The methodologies of swabbing, bacterial isolation and species identification were described previously by Mateus *et al*. (2002) and Silva *et al*. (2010). In addition to the standard procedures used for *E. coli* identification, PCR assays to detect the presence of the *E. coli* 16S rRNA gene was also performed for each strain using specific primers described by Chen *et al*. (2003). The colony morphology of each *E. coli* strain [smooth (S), mucoide (M), non-hemolytic (NH) and hemolytic (H)] was registered after a 24 h incubation at 37ºC in 5% sheep blood agar.

### 3.2. Detection of virulence factors genes and phylogenetic groups

Total *E. coli* DNA was extracted using the DNA mini kit (Qiagen, GmbH, Hilden,Germany). Phylogenetic group was determined by PCR (Clermont et al., 2000) *E. coli* J96 and *E. coli* ATCC 25922 were used as positive controls of these PCR reactions. A

conventional PCR protocol was used to detect the presence of 33 VF-genes (Table 7). The specific primers for amplification of these VF-genes as well as the PCR conditions used in this study were described by others (Table 7). Reference strains *E. coli* J96 (positive for *hlyA, papEF, sfa/focDE, cnf1, fimH, kpsMT-III, papC, fimA, csgA, tcpC, fepA, sitA, iroN* and *traT*), KS52 (positive for *iucD, afa/draBC* and *hlyE*), CECT 685 (positive for *astA* and *kpsMT-II*), CECT4782 (positive for *stx1, stx2* and *eaeA*), ATCC25922 (positive for *focG, usp* and *csgD*), Utrecht 1309 (positive for *f41, f5* and *astA*) and 536 (positive for *sfaS*) were used as positive controls of the PCR reactions. Other strains from our tested collection such as *E. coli* pyo 29 (positive for *ibeA* and *fyuA)*, *E. coli* met 212 (positive for *f17 fimbriae*) and *E. coli* met 12 (positive for *cdtABC*) were also used as positive controls after confirmation of PCR products by DNA sequencing. The boolean data matrix (presence/absence of VF-gene) of 33 characteristics and 49 operational taxonomic units (OTUs) was analyzed by hierarchic numerical methods using the software package BioNumerics (version 6.6, Applied Maths, Kortrijk, Belgium). Resemblance was computed with simple matching coefficient and agglomerative clustering was performed with the unweighted average linkage (UPGMA).

**Table 7 - Comparative prevalence of virulence factor genes in *E. coli* isolates from canine pyometra and bovine metritis origins**

| | | Nº (%) isolates | | |
|---|---|---|---|---|
| **Virulence factores (reference of primers)** | **Gene** | **Canine isolates (n=29)** | **Bovine isolates (n=20)** | **p value** |
| **Adhesins** | | | | |
| Type I fimbriae (Johnson & Stell, 2000b) | *fimH* *fimA* | 29 (100) | 20 (100) | |
| P fimbriae (Johnson & Stell, 2000b) (Yamamoto *et al*., 1995) | *papEF* | 13 (44.8) | 0 (0) | < 0.01 |
| | *papC* | 13 (44.8) | 2 (10) | < 0.05 |
| S + F1C (Johnson & Stell, 2000b) | *Sfa/focDE* | 22 (75.9) | 0 (0) | < 0.00001 |
| F1C fimbriae (Johnson & Stell, 2000b) | *fogG* | 19 (65.5) | 0 (0) | < 0.00001 |

**Table 7 (continuation) - Comparative prevalence of virulence factor genes in *E. coli* isolates from canine pyometra and bovine metritis origins**

| | | Nº (%) isolates | | |
|---|---|---|---|---|
| **Virulence factores (reference of primers)** | **Gene** | **Canine isolates (n=29)** | **Bovine isolates (n=20)** | **p value** |
| **Toxins** | | | | |
| Hemolysin E (Silva *et al*., 2009b) | *hlyE* | 3 (10.3) | 20 (100) | < 0.00001 |
| Alpha-hemolysin (Yamamoto *et al*., 1995) | *hlyA* | 15 (51.7) | 2 (10) | < 0.01 |
| Cytotoxic necrotizing factor 1 (Yamamoto *et al*., 1995) | *cnf1* | 15 (51.7) | 0 (0) | < 0.00001 |
| Cytolethal distending toxins (Yamamoto *et al*., 1995) | *cdtABC* | 0 (0) | 2 (10) | |
| Heat-stable cytotoxin associated with enteroaggregative *E. coli* (Yatsuyanagi *et al*., 2003) | *astA* | 8 (27.6) | 4 (20) | |
| **Iron acquisition systems** | | | | |
| Hem transport protein (Clermont *et al*., 2000) | *chuA* | 27 (93.1) | 1 (5) | < 0.00001 |
| Yersiniabactin system (Johnson & Stell, 2000b) | *fyuA* | 29 (100) | 8 (40) | < 0.00001 |
| Periplasm iron binding protein (Mellata *et al*., 2010) | *sitA* | 29 (100) | 18 (90) | |
| Iron-enterobactin outer membrane transporter (Mellata *et al*., 2010) | *fepA* | 24 (82.8) | 20 (100) | |
| Catecholate siderophore receptor (Mellata *et al*., 2010) | *iroN* | 23 (79.3) | 16 (80) | |
| Aerobactin system (Yamamoto *et al*., 1995) | *iucD* | 3 (10.3) | 8 (40) | < 0.05 |

**Table 7 (continuation) - Comparative prevalence of virulence factor genes in *E. coli* isolates from canine pyometra and bovine metritis origins**

| | | Nº (%) isolates | | |
|---|---|---|---|---|
| **Virulence factores (reference of primers)** | **Gene** | **Canine isolates (n=29)** | **Bovine isolates (n=20)** | **p value** |
| **Protectins** (Johnson & Stell, 2000b) | | | | |
| Serum survival factor | *tratT* | 22 (75.9) | 20 (100) | |
| Microcin CoIV | *cvaC* | 8 (27.6) | 7 (35.0) | |
| Group II capsular Polysaccharide systhesis | *kpsMT-II* | 12 (41.4) | 0 (0) | < 0.00001 |
| Group III capsular Polysaccharide systhesis | *kpsMT-III* | 1 (3.5) | 0 (0) | |
| **Other virulence factors** | | | | |
| Uropathogenic specific protein (Erjavec at al., 2010) | *usp* | 14 (48.3) | 0 (0) | < 0.00001 |
| Invasion of brain endothelium (Johnson and Stell, 2000b) | *ibeA* | 20 (69.0) | 0 (0) | < 0.0001 |
| TIR domain-containing protein (Erjavec at al., 2010) | *tcpC* | 11 (37.9) | 0 (0) | < 0.01 |

### 3.3. Pulsed-field gel electrophoresis

A standard PFGE technique was performed according to Hunter *et al*. (2005). Bacteria were lysed in agarose cubes and genomic DNA was digested with XbaI enzyme (Invitrogen, Carlsbad, CA). The Lambda DNA-PFGE marker (New England Biolabs, Ipswich, England) was added as a molecular size marker on three lanes of each gel. The gel was run in a clamped homogenous electric field (CHEF) system (BioRad, CHEF DR III System) under the PulseNet standard electrophoresis conditions for *E. coli*.

Bionumerics software (version 6.6, Applied Maths, Kortrijk, Belgium) was used to normalize patterns, analyse densitometric curves, calculate Pearson product moment correlation coefficient (r) and perform clustering analysis of DNA profiles using the UPGMA as the agglomerative clustering. The reproducibility of the methodologies was calculated by determining the average similarity value between duplicate pairs of 10% replicates (independent preparation of DNA plugs).

### 3.4. Antimicrobial susceptibility tests

The susceptibility to antimicrobials was investigated using MIC-based broth microdilution panels for pathogenic bacteria (VetMic, National Veterinary Institute, Uppsala, Sweden) for large animals (VetMiC Large Animals, ref. 395105) in the case of bovine *E. coli* isolates, and for small animals (VetMic Small animals, ref. E395122) in the case of canine *E. coli* isolates. *E. coli* ATCC 25922 was used as a quality control. Isolates were classified as susceptible or non-susceptible (intermediate plus resistant results) according to clinical breakpoints values in CLSI (2008) guidelines. The clinical breakpoints values are shown in Table 8.

### 3.5. Statistical analysis

The prevalence of VF genes from *E. coli* isolates of canine and bovine origin were compared using the Fisher's exact test. Associations between VF-genes were analyzed using the correlation matrices function of Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA, 2004). Statistical significance was set at $p<0.05$.

## 4. Results

### 4.1. Colony morphology of *E. coli* isolates

Most (90%) bovine *E. coli* strains showed non-hemolytic and smooth colony morphology (NH+S) and the remaining (10%) showed hemolytic and smooth colony morphology (H+S). Canine *E. coli* strains, exhibited more diverse colony morphology: 45% H+S

phenotype, 28% NH+S phenotype, 21% NH+M phenotype and 7% H+M phenotype. Overall, 52% of canine *E. coli* strains exhibited hemolytic phenotype and 28% exhibited mucoide phenotype. The primary culture plate from two bitches yielded both mucoid/non-hemolytic and smooth/non-hemolytic colonies (Pyo 14 and Pyo 30). In each case both types were chosen for analysis.

### 4.2. Phylogenetic grouping

Bovine isolates belonged to the phylogenetic groups A (25%) and B1 (70%), with only one isolate (5%) belonging to group D. In contrast, the majority of canine isolates belonged to group B2 (93%), and two isolates were assigned to group A (7%).

### 4.3. Characterization of virulence factor gene profiles

All canine and bovine *E. coli* isolates that exhibited α-hemolysis on sheep blood agar carried the gene hlyA. Compared to bovine isolates, canine isolates had more VF- genes (23 (69.7%) versus 15 (45.5%); $p< 0.05$), and higher average number of VF-genes per isolate (13 versus 8, $p<0.05$).

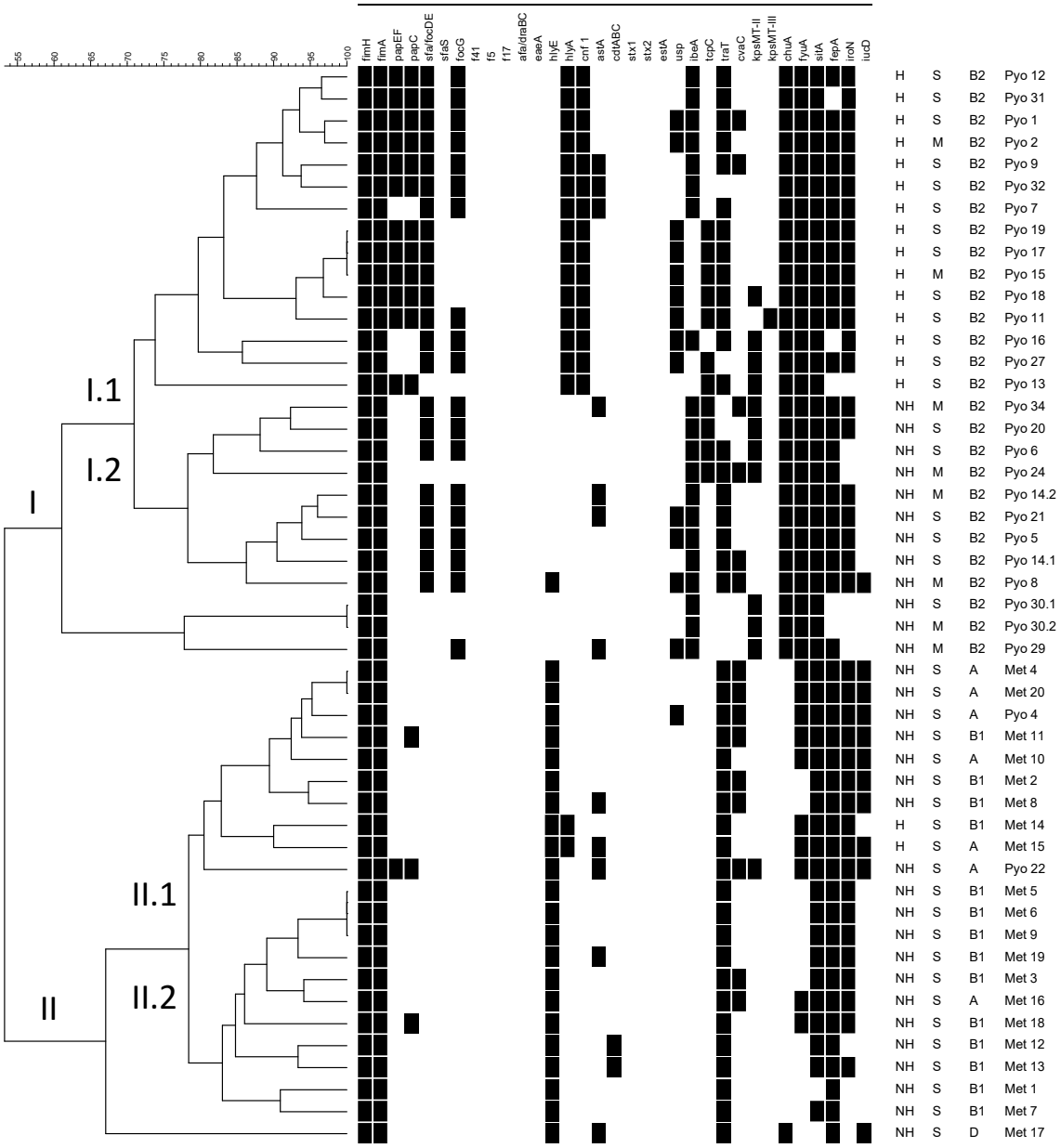
All *E. coli* isolates were negative for *stx1, stx2, estA, eaeA, afa/draBC, sfaS, f41, f5* and *f17* genes. The virulence factor genes *sfa/focDE, focG, cnf1, kpsMT-II, kpsMT-III, usp, ibeA*, *tcpC* were specific of canine isolates and *cdtABC* was detected only in bovine isolates. The comparative prevalence of VF-genes in bovine and canine isolates is shown in Table 7. Canine isolates positive for *hlyA* gene (n=15) were also positive for cnf1 gene ($r=1$, $p<0.0001$), and positive for *papEF/papC* genes in 80% (n=12) of occasions ($r=0.73$, $p<0.0001$). Canine *E. coli* isolates harbored more genes for iron-acquisition systems than bovine isolates. An association of 5 (*sitA-fuyA-chuA-fepA-iroN* or *iucD*), 4 (*sitA-fuyA-chuA-fep* or *iroN*) and 3 (*sitA-fuyA-chuA*) genes was present in 20 (69%), 5 (17%) and 3 (10%) *E. coli* canine isolates, respectively. In contrast, only 8 bovine isolates (40%) harbored 4 iron-acquisition genes (*sitA-fepA-iroN- iucD* or *chuA*), and a further 8 (40%) isolates harbored 3 of these genes (*sitA-fepA-iroN*). The remaining isolates harbored one (n=1) or two (n=3) iron-acquisition genes.

Results of the cluster analysis based on virulence profile are shown in Fig. 12. Two major virulence clusters were identified. Cluster I included all canine isolates assigned to phylogenetic group B2 (n=27), encoding an average of 13 VF-genes per isolate. This cluster could be further divided into two sub-clusters based on the presence (cluster I.1) or absence (cluster I.2) of *hlyA, cnf1* and *papC/EF* genes. Cluster II included all bovine isolates and the two canine isolates belonging to phylogenetic group A, encoding an average of 8 VF-genes per isolate. This cluster could be divided into two main sub-clusters based on the prevalence of *cvaC, fyuA* and *iucD* genes (higher in cluster II.1 compared to cluster II.2).

### 4.4. PFGE analysis

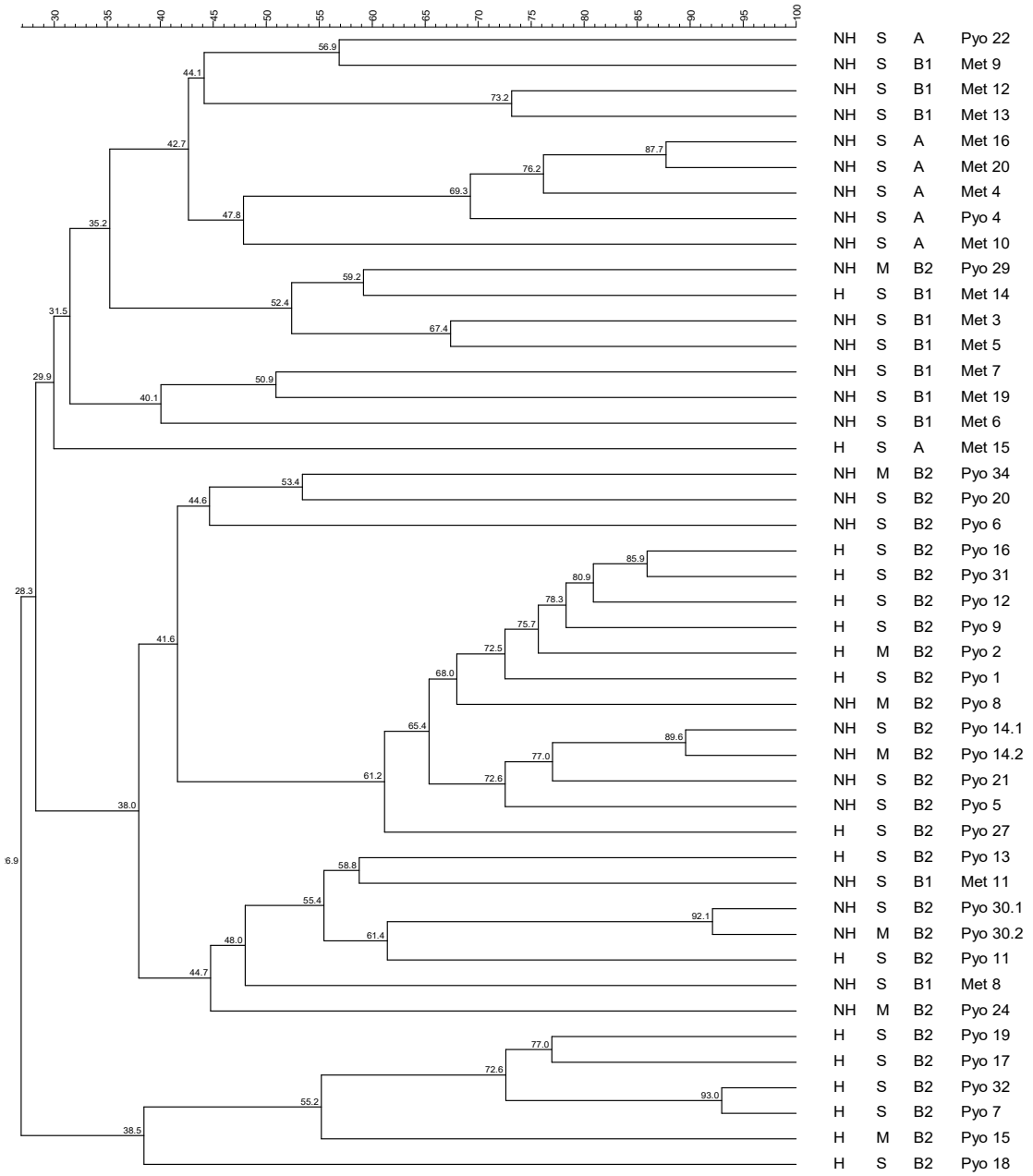
Only 16 bovine *E. coli* isolates were typeable by PFGE. The remaining four strains (Met 1, 2, 17 and18) had been previously genotyped by ERIC-plus-REP–PCR (Silva et al., 2009a), all being assigned to different clonal types. According to the average reproducibility, the cut-off level for clonal identification was set at 86.1% (Fig. 13). Overall, the degree of genetic similarity between canine and bovine *E. coli* isolates was 28.3%. Only three canine *E. coli* isolates (two from phylogenetic group A and one from phylogenetic group B2) were included in the bovine group, with an average of similarity of 61.9% with the bovine isolates. Conversely, two bovine *E. coli* isolates (from phylogenetic group B1) were present in the canine group, with an average of similarity of 53.4% with the canine isolates (Fig. 13). From the 29 canine *E. coli* isolates, 26 clonal types were identified, while from the 16 bovine *E. coli* isolates 15 clonal types were identified. In the two bitches from which two *E. coli* strains with different phenotypes were isolated, PFGE analysis showed that both strains belonged to the same clonal type (Pyo 14 and Pyo30, with a similarity of 89.6% and 92.1%, respectively) (Fig. 13).

**Figure 12 - Cluster analysis of virulence factor genes profile of *E. coli* uterine isolates of canine pyometra (n=29) and bovine puerperal metritis (n=20) origin**




Two major virulence clusters were identified: Cluster I included all canine isolates assigned to phylogenetic group B2 (n = 27), This cluster could be further divided into two sub-clusters based on the presence (cluster I.1) or absence (cluster I.2) of *hlyA*, cnf1 and *papC/EF* genes. Cluster II included all bovine isolates and the two canine isolates belonging to phylogenetic group A. This cluster could be divided into two main sub-clusters based on the prevalence of *cvaC, fyuA* and *iucD* genes (higher in cluster II.1 compared to cluster II.2).

**Figure 13 - PFGE clonal type analysis of *E. coli* uterine isolates of canine pyometra (n=29) and bovine puerperal metritis (n=16) origin**




According to the average reproducibility, the cut-off level for clonal identification was set at 86.1%. The degree of genetic similarity between canine and bovine *E. coli* isolates was 28.3%. Three canine *E. coli* isolates were included in the bovine group, with an average of similarity of 61.9% with the bovine isolates. Two bovine *E. coli* isolates were present in the canine group, with an average of similarity of 53.4% with the canine isolates. From the 29 canine *E. coli* isolates, 26 clonal types were identified, while from the 16 bovine *E. coli* isolates 15 clonal types were identified. In the two bitches from which two *E. coli* strains with different phenotypes were isolated, PFGE analysis showed that both strains belonged to the same clonal type (Pyo 14 and Pyo30, with a similarity of 89.6% and 92.1%, respectively).

### 4.5. Antimicrobial susceptibility test

Table 8 shows the antimicrobial susceptibility of canine and bovine *E. coli* isolates. *E. coli* ATCC 25922 was always susceptible to the ranges of values available in the microdilution panels tested. Twenty (69%) canine isolates were sensitive to all tested antimicrobials. The most frequent non-susceptibilities were observed directed to ampicillin (17%, AMP), and amoxicillin/clavulanic acid (14%, AMC). In contrast, seven (35%) bovine isolates were susceptible to all antimicrobials tested, and all the remaining were sensitive to ceftiofur, gentamicin (GM) and enrofloxacin (ENR). The most frequent non-susceptibilities were directed towards tetracycline (65%, TE), ampicillin (35%), and trimethoprim/sulphamethoxazole (30%, SXT). Four (14%) canine isolates were non-susceptible to more than one antimicrobial: AMP + AMC (n=2); AMP + AMC + cefotaxime + GM (n=1); AMP + AMC + GM + SXT + TE + ENR (n=1). Seven (35%) bovine isolates were non-susceptible to more than one antimicrobial: TE + AMP (n=1); TE + AMP + SXT (n=6). Overall, the prevalence of cases of several non-susceptiblility to antimicrobials were lower in isolates belonging to phylogenetic group B2 than in isolates belonging to phylogenetic groups B1 and A (11% versus 43%, $p < 0.05$).

**Table 8 - Distribution of the minimum inhibitory concentrations (MICs) of antimicrobials for canine (n = 29) and bovine (n = 20) *Escherichia coli* isolates**

| | MIC (mg/L) * | | | | | | | | | NS |
|---|---|---|---|---|---|---|---|---|---|---|
| **Antimicrobial** | 0.12 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | % |
| **Canine Isolates** | | | | | | | | | | |
| Ampicilin | | | | 2 | 17 | 5 | | 5 | | 17 |
| Amoxacilin/Clavulanic acid | | | | | | | 25 | | 4 | 14 |
| Cefotaxim | | | 28 | | 1 | | | | | 3 |
| Gentamicin | | | | | 24 | 1 | 3 | | 1 | 17 |
| Trimethoprim/Sulfamethoxazole | | | 27 | 1 | | 1 | | | | 3 |
| Tetracycline | | | | 21 | 4 | 2 | | 2 | | 7 |
| Enrofloxacine | 28 | | | 1 | | | | | | 3 |
| **Bovine Isolates** | | | | | | | | | | |
| Ampicilin | | | | 1 | 11 | 1 | | 7 | | 35 |
| Ceftiofur | | 18 | 2 | | | | | | | 0 |
| Gentamicin | | | | | 20 | | | | | 0 |
| Trimethoprim/Sulfamethoxazole | | | 14 | | | | 6 | | | 30 |
| Tetracycline | | | | 5 | 2 | | | 13 | | 65 |
| Enrofloxacine | 20 | | | | | | | | | 0 |

*Grey areas indicate the range of concentrations tested for each antimicrobial; MIC values above the tested range are indicated as the value immediately above the range limit. Values equal to or below the tested range are indicated as the lowest tested concentration. Clinical breakpoints values for non- susceptibility are given as vertical bars. NS (%) represents the percentage of isolates non susceptible (intermediate + resistant) to a particular antimicrobial.

## 5. Discussion

In this work we undertook the comparative genotypic and phenotypic characterization of *E. coli* strains isolated from bovine clinical metritis and canine pyometra cases. Overall, our results showed that *E. coli* strains isolated from canine pyometra cases have a significantly higher virulence potential than *E. coli* strains isolated from bovine clinical metritis cases. Bovine and canine *E. coli* isolates presented a low degree of genetic similarity (28%), indicating host species-specific genomic profiles. A high genomic diversity was observed among *E. coli* isolates of both host species. This diversity suggests that in each disease, the isolates do not originate from a specific clone that is epidemically spread between animals, as also suggested by Hagman & Kühn (2002) and Silva *et al.* (2009a). A clear difference was observed in the phylogenetic group distribution between canine and bovine *E. coli* isolates. Almost all canine *E. coli* strains were assigned to group B2, whereas bovine *E. coli* strains were mainly assigned to groups B1 and A. The phylogenetic group distribution of bovine *E. coli* isolates is in agreement with those reported by Sheldon *et al*. (2010) and Son *et al.* (2009), regarding isolates of metritis and fecal origin, respectively. A high prevalence of phylogenetic group B2 was also reported in canine UTI *E. coli* isolates (Mateus et al., 2013). Canine and bovine *E. coli* isolates showed a distinct VF-gene profile, evidenced by the cluster analysis based on virulence traits. Contrary to bovine metritis strains, canine pyometra strains harbored multiple VF-genes typical of extraintestinal pathogenic *E. coli*. Genes encoding for Type 1 fimbriae were present in all bovine and canine *E. coli* isolates. However, in canine strains, genes for other adhesins (P fimbriae, F1C fimbriae) were also present. Adherence to bovine (Sheldon et al., 2010) and canine (Krekeler et al., 2013) endometrial cells was shown to be mediated at least in part by Type 1 fimbriae, although targeted deletion of specific adhesin genes in a canine pyometra strain was compensated by the presence of

other adhesins, which may indicate functional redundancy among adhesins (Krekeler et al., 2013). In bovine *E. coli* strains VF-genes encoding toxins were either absent or presented a low prevalence. Therefore their role in the pathogenesis of clinical metritis is probably limited (Sheldon et al., 2010; Silva et al., 2009a). Canine *E. coli* strains had a high prevalence of the hlyA and cnf1 genes. Similar prevalences in pyometra *E. coli* isolates were reported by others (Chen et al., 2003; Siqueira et al., 2009). These toxins can be associated to the tissue damage and increased inflammation observed in pyometra uteri, and may also favor bacterial invasion by killing immune cells (reviewed by Bien et al., 2012). Although hlyE gene was present in all bovine isolates, its role in metritis is unclear, as similar prevalence were observed in *E. coli* strains recovered from clinical metritis and normal puerperium cows (Silva et al., 2009a). HlyE is involved in the mechanism of bacterial iron acquisition. The detection of multiple iron acquisition genes in the majority of the isolates (≥ 80) is associated with the fact that the uterus is an iron limiting environment. During infection, the amount of iron available to bacteria is even lower due to the production of host proteins that interact with iron metabolism. The iucD gene prevalence was higher in bovine (40%) than in canine (10%) isolates, indicating that this siderophore is an important iron acquisition system in the bovine uterus environment. However, a similar prevalence of the iucD gene was observed in bovine *E. coli* strains isolated from clinical metritis and normal puerperium cows (Silva et al., 2009a). In contrast with the iucD gene, the iron acquisition genes fyuA and chuA had a higher prevalence in canine (100% and 93%, respectively) than in bovine (40% and 5%, respectively) *E. coli* isolates. Besides its role in iron uptake, yersiniabactin also interferes with the host response and innate immune system, being characteristic of bacteriemic *E. coli* (Johnson & Stell, 2000b). Therefore, the above genes may represent a relevant component of the virulence of canine pyometra *E. coli* strains, contributing to the persistence and load of bacteria. With the exception of traT protectin encoding gene, with a high prevalence in both groups, kpsMT-II, ibeA, usp and tcpC genes and mucoide phenotype were only present in canine *E. coli* strains. These VF-genes may have relevant roles in the virulence potential of the bacterium and the pathogenesis of pyometra, by promoting endothelial cell invasion and interfering or avoiding the host defense mechanisms (Johnson & Stell, 2000b; Yadav et al., 2010). The most frequent antimicrobial non-susceptibility of canine pyometra *E. coli* strains were towards

amoxacillin/clavulanic acid (14%) and ampicillin (17%), the most commonly used antimicrobial drugs for the treatment of uncomplicated cases of canine pyometra. A similar prevalence of resistance to the above antimicrobials was reported by Hagman & Greko (2005). Only 3% of the canine *E. coli* isolates were not susceptible to enrofloxacin, an antimicrobial currently used for treatment of complicated pyometra cases and UTIs. Since UTI and pyometra may co-exist in the same animal, enrofloxacin may be the antimicrobial of choice before antimicrobial susceptibility is confirmed. In the puerperal cow, tetracycline is widely used for both therapeutic and prophylactic purposes. This is probably the main reason for the occurrence of the low susceptibility observed by us (35%) and others (Sheldon et al., 2004b). It was demonstrated that commensal enteric *E. coli* from healthy lactating cows are important reservoirs for tetracycline and ampicillin resistance determinants (Sawant et al., 2007). The high susceptibility of bovine *E. coli* strains to ceftiofur reflects the good efficacy this antimicrobial in the treatment of metritis and clinical endometritis (Galvão, Greco, Vilela, Sá Filho, & Santos, 2009; Sheldon et al., 2004b). The prevalence of resistance towards two or more antimicrobials was lower in *E. coli* strains belonging to phylogenetic group B2 than in strains belonging to phylogenetic groups B1 and A, suggesting a trade-off between resistance and virulence.

The low virulence potential here observed for *E. coli* strains isolated from bovine metritis cases is in accordance to the so far known role of this bacterium in the pathogenesis of metritis and may be related with the short persistence of the bacterium in the uterus during the cow puerperal period, even in the case of metritis (Mateus et al., 2002; Sheldon et al., 2009). Although metritis isolates lack the common pathogenicity genes associated with virulence of ExPEC, we cannot forget that in the postpartum uterus the epithelial barrier is already broken, given open access to bacteria to other endometrial cell components. Also, puerperal cows are characterized by an immunosuppressive state, which predispose to uterine infection. In the bitch, the high endogenous progesterone concentration in the first half of diestrus impairs anti- microbial defenses (Silva et al., 2012) and predisposes to endometrial *E. coli* adherence (Ishiguro et al., 2007). Besides the above factors, the exuberant clinical signs associated with pyometra may be related with the high virulence profile of *E. coli* strains isolated from affected bitches. This virulence potential may enable *E. coli* to be the solo bacterium in the pathogenesis of canine pyometra. It cannot be forgotten the role of endotoxins (LPS) in both diseases. Concentrations of uterine fluid

endotoxin and $PGE_2$ were related to the severity of bovine metritis and absorption of endotoxin from the uterus was associated with ovarian dysfunction (Mateus et al., 2003). In bitches with pyometra, high blood concentration of endotoxin has been related to poor prognosis (Hagman, 2012).

In conclusion, data here presented shows that *E. coli* strains isolated from canine pyometra cases are genetically distinct from those isolated from bovine puerperal metritis cases. Canine pyometra *E. coli* strains exhibit a higher virulence profile than bovine metritis strains, which may partially explain the different clinical outcomes of the uterine infection in these two species. Non-susceptibility to antimicrobials currently used in the treatment of both diseases was low. However, non-susceptibility of bovine *E. coli* strains to tetracycline was high, probably reflecting the inadequate past use of this antimicrobial.

## CHAPTER VI - IMMUNOMODULATION IN THE CANINE ENDOMETRIUM BY UTEROPATHOGENIC *ESCHERICHIA COLI*

Henriques, S.[1], Silva, E.[1], Silva, M.F., Carvalho, S., Diniz, P., Lopes-da-Costa, L., Mateus, L. (2016). Submitted to *Veterinary Research*

[1]These authors contributed equally to this work

Contribution of Sofia Henriques to this article:

Sofia Henriques participated in the laboratory work and data analysis process and co-produced the manuscript.

## 1. Abstract

This study was designed to evaluate the role of *E. coli* α-hemolysin in the pathogenesis of canine pyometra, and on the immune response of canine endometrial epithelial and stromal cells. In Experiment 1, the clinical, hematological, biochemical and uterine histological characteristics of β-hemolytic and non-hemolytic *E. coli* pyometra bitches were compared. β-hemolytic *E. coli* pyometra was associated with the occurrence of metritis and with an higher uterine tissue damage than non-hemolytic *E. coli* pyometra. β-hemolytic *E. coli* pyometra endometria had higher gene transcription of *IL-1β* and *IL-8* and lower gene transcription of *IL-6* than non-hemolytic pyometra endometria. In Experiment 2, the immune response of endometrial epithelial and stromal cells, to hemolytic and non-hemolytic *E. coli* strains (wild-type and hemolytic strain with deleted α-hemolysin gene) were compared. The cytotoxic effect of α-hemolysin was more severe in endometrial stromal than in epithelial cells. Endometrial stromal cell damage by α-hemolysin is a potential relevant step of *E. coli* virulence in the pathogenesis of pyometra. Additionally, inhibition of cytokine production by α-hemolysin, which may occur during the early stages of infection, may allow bacteria to establish a niche prior to the activation of an adequate innate immune response. Low levels of pro-inflammatory cytokines IL-1β and TNFα may compromise the chemotaxis and activation of immune cells, leading to a precocious high level of cell and tissue damage. Down-regulation of IL-10 production might be associated with an excessive inflammatory reaction, thus enhancing tissue damage.

**Keywords:** canine pyometra, *Escherichia coli*, HlyA, endometrial cells

## 2. Introduction

Pyometra is a common diestrous disease of bitches. *E. coli* is the bacterium most frequently isolated from the pyometra uterus (Mateus et al., 2013), and its presence is associated with severe systemic signs and a potentially life-threatening condition. The SIRS is detected in more than 50% of bitches with *E. coli* pyometra (Karlsson et al., 2012). These *E. coli* isolates derive from the host's fecal and perineal flora (Hagman & Kühn, 2002), being mainly assigned to phylogenetic group B2, and characterized by a high number of UPEC VF genes PAIs markers (Mateus et al., 2013). The *hlyA* VF gene was detected in 48% of pyometra *E. coli* strains, whereas in *E. coli* isolates from urinary tract infection and from fecal origin detection only attained 30% and 19%, respectively markers (Mateus et al., 2013). HlyA is a RTX pore- forming exotoxin. Depending on its concentration and on the type of targeted cells, HlyA either displays cytolytic activity on erythrocytes and nucleated host cells, or induce innate immune signaling pathways (reviewed by Wiles & Mulvey, 2013). However, its role in the pathogenesis of pyometra is unknown.

TLR-mediated immune surveillance is an important component of the defense mechanisms within the canine uterus (Silva et al., 2012). Up-regulation of TLR 2 and 4 transcription (Hagman et al., 2009; Silva et al., 2010) and expression (Silva et al., 2012) was observed in *E. coli* pyometra endometrium. Uterine inflammatory response towards *E. coli* is associated with an endometrial up-regulation of genes related with chemokines, cytokines, inflammatory cell extravasation, anti-bacterial action, proteases and innate immune response (Hagman et al., 2009). In accordance, pyometra is characterized by endometrial tissue damage, infiltration by inflammatory cells, accumulation of pus, and increased expression of inflammatory mediators such as interleukins IL-1β, IL-6 and IL-8 (Hagman et al., 2009; Voorwald et al., 2015). Different expression patterns of cytokines were observed in bitches with or without pyometra-associated SIRS (Karlsson et al., 2012).

Most of the studies on canine pyometra were carried out at the time of diagnosis, a late stage of the disease, and did not take into account the virulence background of *E. coli*. The characterization of the endometrial cell cytokine response to *E. coli* may lead to a relevant insight into the pathogenesis of pyometra. Additionally, the characterization of the role of

specific *E. coli* VF genes in the modulation of the endometrial immune response and on the pathogenicity of the bacterium may prove rewarding in the development of novel diagnostic and therapeutic approaches of the disease. In this regard, α- hemolysin becomes a promising candidate.

This study includes two experiments. Experiment 1 was designed to evaluate and compare the clinical and uterine histological and immune response gene transcription of hemolytic and non-hemolytic *E. coli* pyometra bitches. Prompted by results of Experiment 1, Experiment 2 was designed to evaluate the effects of *E. coli* HlyA on the modulation of the inflammatory response of canine endometrial epithelial and stromal cells.

## 3. Materials and methods

### 3.1 Experiment 1

Healthy diestrous (n=10) and pyometra (n=18) bitches presented to the Hospital of the Faculty of Veterinary Medicine of the University of Lisbon were selected for this experiment. The average age of bitches was 8 years (range 3 – 13 years). Healthy bitches were submitted to OVX for contraceptive purposes, as requested by owners. In pyometra bitches the diagnosis was based on case history, clinical signs and the ultrasonographic finding of an enlarged, fluid-filled uterus. A blood sample for hematologic (Hemogram) and biochemical (urea, creatinine, phosphatase alkaline, alanine-aminotransferase) analysis was collected prior to OVX. After removing the uterus, an intra-uterine swab was processed for bacteriological analysis. Selection of the 18 pyometra bitches was based on the isolation of hemolytic (n=8) and non-hemolitic (n=10) *E. coli* strains. Uterine samples were collected as described previously (Silva et al., 2012) and either fixed for 24h in 4% neutral phosphate buffered formalin (for IHC, described in 2.1.1) or immersed for 24h in RNAlater (Qiagen, GmbH, Hilden, Germany) and then stored at −80ºC (for RT-PCR and qRT-PCR, described in 2.2.4).

### 3.1.1 Immunohistochemistry

IHC was used to identify T lymphocytes (Rabbit polyclonal anti-human CD3, diluted 1:200; Dako, Glostrup, Denmark), B lymphocytes (mouse monoclonal anti-human anti-CD79 αcy, clone HM57, diluted 1:150; Dako) and granulocytes and macrophages (mouse anti-human MCA874G, clone MAC387, diluted 1:400, Dako) in uterine samples. Except for anti-CD3 antibody, all protocol steps were carried out using the Novolink Polymer Detection System (Novocastra, Leica Biosystems, Newcastle, UK), according to the manufacturer's instructions. The antigen retrieval step was performed by microwave treatment (3×5 min) in Tris–EDTA buffer (pH 9.0). After endogenous peroxidase blocking and treatment with protein block solution (*Protein Block Solution*- Kit NovoLink$^{TM}$), sections were incubated 1h at room temperature with the respective primary antibodies. CD3 immunostaining was carried out as previously described (Silva et al., 2012) with minor modifications. Briefly, endogenous peroxidase was quenched by incubating the slides in 3% hydrogen peroxide in water for 30 min followed by antigen retrieval in Tris–EDTA buffer (pH 9.0), as described above. Blocking was performed with blocking solution (PBS + 0.1% Tween + 5% goat serum + 2.5% BSA), for 1h at room temperature followed by incubation with the primary antibody for 2h. The peroxidase conjugated goat anti-rabbit IgG polyclonal antibody (diluted 1:100, Dako) was used as secondary antibody. For all antibodies, the staining was developed by incubating the slides with the substrate diaminobenzidine (DAB kit, Zytomed Systems, Berlin, Germany). Staining without the primary antibody and staining with the isotype-matched irrelevant monoclonal antibody were used as negative controls. Human tonsil and dog spleen sections were used as positive control tissues.

## 3.2 Experiment 2

### 3.2.1 Cell culture

Uteri were obtained from healthy bitches (1 to 3 years) submitted to OVX for contraceptive purposes at the Teaching Hospital of the Faculty of Veterinary Medicine of the University of Lisbon. Only uteri from the first half of diestrus (as determined by

vaginal cytology, blood progesterone concentrations and histology), without histological evidence of CEH and a negative bacteriological result were allocated to the study.

Endometrial epithelial and stromal cell populations were isolated as described by Bläuer, Heinonen, Matikainen, Tomás & Ylikomi (2005) and Stadler, Handler, Schoenkypl & Walter (2009) with some modifications. Briefly, small endometrial strips were digested with 1mg/ml of collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) and 5µg/ml of DNAse I (Sigma-Aldrich, St. Louis, EUA) in RPMI-1640 (GIBCO ®, Invitrogen Corporation, New York, EUA) supplemented with gentamicin (50µg/ml, Sigma-Aldrich) and anfotericin B (2.5µg/ml, Sigma-Aldrich). After 60-90 minutes of incubation with gently shaking at 37ºC, the cell suspension was filtered through a sterile mesh to remove undigested tissue. The suspension was centrifuged at 200 x g for 5min and re-suspended in 10ml of culture medium (RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum, 50µg/ml of gentamicin and 2.5µg/ml de anfotericin B). Epithelial organoids and stromal fibroblasts were separated by differential centrifugation and filtered through a sterile 40µm pore size filter. Stromal suspension (bottom of the filter) was pelleted by centrifugation (250 x g), freed of red blood cells and suspended in 1ml of culture medium. Further purification of stromal cells was done by gently pipetting the fraction onto 9ml of culture medium and letting them to sediment by gravity for 30min.

Organoid suspension was obtained after back washing the filter with culture medium. After 2 steps of sedimentation by gravity for 5min, epithelial cells suspension was centrifuged (200 x g,5 min) and the pellet was suspended in 3ml of trypsin 0.25% - EDTA (GIBCO ®) with 5µg/ml of DNAse I. After 2-5min of incubation, organoids were unbundled with a 26G needle and 2ml syringe in order to get a single-cell suspension. The viability of the stromal and epithelial cells obtained was 80-95% as assessed by Trypan Blue exclusion dye staining.

Stromal and epithelial cells were seeded in 24-well plates at a density of $1x10^5$cells/ml and $2x10^5$cells/well/ml, respectively, and cultured in 1ml of culture medium supplemented with Insulin-Transferrin-Selenium (1x) (ITS liquid media supplement, 100X, Sigma-Aldrich). For stromal cells, medium was changed 12h after plating to allow selective attachment. For epithelial cells, medium was removed after 24h to discard nonattached cells. Culture media was then changed every 48h until cells reached 90-95% confluence.

The purity of stromal and epithelial cells was determined by their morphology after staining with Giemsa (Accustain ®, Sigma-Aldrich) in glass coverslips.

### 3.2.2 *E. coli* strains

The two *E. coli* strains used in this study, Pyo14 and Pyo18, were previously isolated from the uterus of pyometra bitches and characterized (Henriques, Silva, Lemsaddek, Lopes-da-Costa, & Mateus, 2014). Although both strains were from phylogenetic group B2, only Pyo18 carries *hlyA* gene and have a hemolytic phenotype in sheep blood agar characterized (Henriques et al., 2014). The isogenic *hlyA* deletion mutant was generated in *E. coli* Pyo18 strain using the Lambda-Red recombinase system as described by Datsenko & Wanner (2000). Briefly, the chloramphenicol cassette was amplified from a PKD3 plasmid using primers with 40 nucleotide extension homologous to the *hlyA* gene (*hlyA*1: 5′-cagatttcaatttttcattaacaggttaagaggtaattaagtgtaggctggagctgcttc-3′; *hlyA*2:5′-cagcccagtaag attgctattatttaaattaataaaatgggaattagccatggtcc-3′). The following PCR conditions were used: initial denaturation of 5 min at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 50°C, 2 min at 72°C and a final extension of 5 min at 72°C. A purified PCR product was used to replace the chromosomal *hlyA* gene in Pyo18 strain using the helper plasmid pKD46 expressing the lambda recombinase. For this purpose, Pyo18 strain was previously transformed with pKD46 plasmid, which after induction with 0.01ML-arabinose promotes homologous recombination between the PCR product and the chromosomal target gene. The PCR product was electroporated into Pyo18/PKD46 and the chloramphenicol resistance gene was eliminated using the helper plasmid pCP20. Elimination of pCP20 plasmid was accomplished by bacterial growth at 43ºC. The *hlyA* gene deletion was confirmed by PCR analysis (Δ*hlyA1*: 5′-ccattagaggttcttgggc- 3′; Δ*hlyA2:* 5′-ggaataaaccaggtaaagtc- 3′) and DNA sequencing.

Before stimulation experiments, bacteria were plated onto Columbia agar medium and incubated at 37ºC overnight. For each bacterium, one bacterial colony was inoculated into liquid LB medium at 37ºC for 48h without agitation, washed three times by centrifugation (5000 x g for 5min) and re-suspended in sterile PBS. The bacterial suspension was diluted at a final working concentration equivalent to $10^8$ CFU/ml. Similar bacterial growth curves were obtained for the three *E. coli* strains.

### 3.2.3. *E. coli* cell stimulation

Cells were pre-incubated in medium without antibiotic (RPMI-1640 supplemented with 10% of FBS) during 24h before bacteria inoculation. Endometrial epithelial and stromal cells were incubated with the *E. coli* isolates [multiplicity of infection (MOI) of 5-10], LPS (1µg/ml *E. coli* O55:B5, Sigma-Aldrich) or medium alone for 1h and 4h at 37ºC (adhesion step). Cells were washed with PBS to remove non-adherent *E. coli* and further incubated for 1.5h with fresh medium containing 50µg/ml gentamicin to eliminate extracellular bacteria (internalization step). The medium was then removed and the cells washed three times with PBS. Cells of two duplicate wells were lysed with 350µl of RLT/b-ME buffer (*Rneasy mini kit*, Qiagen GmbH, Hilden, Alemanha) and frozen at -80ºC until RNA extraction. Parallel plates were used to assess adherence and internalization levels, as described (Letourneau, Levesque, Berthiaume, Jacques, & Mourez, 2011). Experiments were repeated on at least three independent occasions, with treatments applied to duplicate wells. Results on morphology and number of cells are those obtained after the internalization step and are relative to the unstimulated control.

### 3.2.4 RNA extraction, cDNA synthesis, RT-PCR and qRT-PCR

Total RNA was isolated from pyometra endometrial tissue and from cell cultures using the Rneasy mini kit (Qiagen GmbH, Hilden, Germany) and DNA digestion was performed with the RNase-free DNase set (Qiagen GmbH). RNA concentration and purity was determined in a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, Walthman, USA). cDNA synthesis was performed by reverse transcription of 500ng (endometrial tissue) or 400ng (cell cultures) of total RNA using the SuperScript III First-Strand synthesis SuperMix for qRT-PCR (Invitrogen, Austin, TX, USA), according to the manufacturers' protocols. RT-PCR and qRT-PCR was performed as described by Silva *et al*. (2012). In Experiment 1, RT-PCR was performed to screen the presence of transcripts of TLR4 pathway components in endometrial tissue. qRT-PCR was performed to quantify the mRNA levels of cytokines in endometrial tissue (see 3.2). In Experiment 2, only qRT-PCR was performed to quantify mRNA expression in endometrial cells. Primers (Tables 9 and 10) were first chosen with Primer3 Software and for qRT-PCR confirmed with Primer

Express® Software (Applied Biosystems, Foster City, CA, USA). Ribosomal protein L27 gene (RPL27) was chosen as the housekeeping gene. Real-time PCR was performed in duplicate wells on StepOnePlus™ (Applied Biosystems, Foster City, CA, USA), using the universal temperature cycles as suggested by the manufacturer. Melting curves were acquired to ensure that a single product was amplified in the reaction. All PCR reactions were carried out in 96-well optical reaction plates (Applied Biosystems, Warrington, UK) with 1X Power SYBR® Green PCR Master Mix (Applied Biosystems), 1ng (endometrial tissue) or 2ng (cell cultures) of cDNA, 80 nM of each primer in a total reaction volume of 12.5µl. After analyzing the melting curves, the PCR products were run through a 2.5% gel agarose to confirm the expected product size. The identity of PCR products was initially confirmed by DNA sequencing. The data of relative mRNA quantification was analyzed with the real-time PCR Miner algorithm (Zhao & Fernald, 2005). Results of qRT-PCR are expressed as the fold increase relative to the unstimulated control.

**Table 9 - Primer sequences used for RT-PCR**

| Target Gene | Sequence (5`-3`) | Annealing temperature (ºC) | Product size (bp) | GeneBank accession number |
|---|---|---|---|---|
| *MyD88* | FW- ACTATCGGCTGAAGTTGTGTGTGT<br>RV- TGGTGTAGTCACAGACAGTGATGAA | 58.3 | 276 | XM_534223 |
| *TRAM* | FW- GGGTGTCAGGAAGTCGAAAATA<br>RV- CTGCGTGCAGTATCACAAACTT | 56.8 | 266 | NM_001204337 |
| *TRAF6* | FW- AAACTGTGAAAACAGCTGTGGA<br>RV- CAGTTCATGCAAGAAACCTGTC | 56.1 | 564 | XM_003432322 |
| *TRIF* | FW- CTTCCAAAGCCCATAGAGGA<br>RV- AAGCGGTGTCTTCTACAGGAA | 55.4 | 487 | XM_849573 |
| *IRF3* | FW- GGTGCCTACACTCCTGGAAA<br>RV- CTTCATCAGGCACCAAGAGC | 56.0 | 418 | XM_005616307 |
| *NFkB* | FW- TGTTTCACTTGGATCCTTTGAC<br>RV- AGATCCCATCCTCACAGTGTTT | 55.4 | 327 | NM_001003344 |
| *IL-1β* | FW- CACCAGTGAAATGATGGCTTAC<br>RV- CTCATGTGGAACACCACTTGTT | 56.0 | 453 | NM_001037971 |
| *IL-6* | FW- GTACATCCTCGGCAAAATCTCT<br>RV- GGATGAGGTGAATTGTTGTGTG | 56.0 | 410 | NM_001003301 |
| *IL-8* | FW- TTGGCAGCTTTTGTCCTT<br>RV- GGGCCACTGTCAATCACT | 52.5 | 149 | NM_001003200 |
| *IL-10* | FW- AAGCTGGACAACATACTGCTGA<br>RV- TGTCAAACTCACTCATGGCTTT | 56 | 316 | NM_001003077 |

**Table 9 (continuation) - Primer sequences used for RT-PCR**

| Target Gene | Sequence (5`-3`) | Annealing temperature (ºC) | Product size (bp) | GeneBank accession number |
|---|---|---|---|---|
| *TNF-α* | FW- TGACAAGCCAGTAGCTCATGTT<br>RV- CGGCAAAGTCCAGATAGTTAGG | 56 | 410 | NM_001003244 |
| *IL-2* | FW- TTGTCGCAAACAGTGCACCTA<br>RV- CCTGGAGAGTTGGGGGTTCT | 60 | 131 | NM_001003305 |
| *IL-4* | FW- CTCACCAGCACCTTTGTCCA<br>RV- GTCAGCTCCATGCACGAGTC | 60 | 107 | NM_001003159 |
| *IFN-β* | FW- CAGTAGATGCATCCTCCAAACA<br>RV- GACTATTGTCCAGGCACAGATG | 55.9 | 493 | NM_001135787 |
| *IFN-γ* | FW- GCTGTAACTGTCAGGCCATGTTT<br>RV-TGTTTTGTCACTCTCCTCTCTCCA | 60 | 140 | NM_001003174 |

**Table 10 - Primers sequences used for quantitative real time PCR (qRT-PCR)**

| Target Gene | Sequence (5′-3′) | Product size (bp) | GeneBank accession number |
|---|---|---|---|
| *NFKB* | FW-GGAGGAGACCGGCAGCTTA<br>RV-GCCGGTGCTATCTGGAAGAA | 129 | NM_001003344.1 |
| *IRF3* | FW -TCACCACGCTACACCCTCTG<br>RV -ATTTCCAGCAGGGCCCTAAG | 119 | XM_005616307.1 |
| *IRF7* | FW- GTCGGGGCTCCCCATACTAC<br>RV- CCCTTCTCGCTGCACGTACT | 143 | XM_005631711.1 |
| *TNF-α* | FW- CTGCCTCAGCCTCTTCTCCTT<br>RV- CTGGGCAAGAGGGCTGATTA | 133 | NM_001003244.4 |
| *IL-1β* | FW-GAAGAAGCCCTGCCCACA<br>RV-AATTATCCGCATCTGTTTTGCAG | 104 | NM_001037971 |
| *IL-1α* | FW- TTGTGAGTGCCCAAAATGAAGA<br>RV- CCATGACGCTCCCAAAAGA | 109 | NM_001003157.2 |
| *IL-6* | FW-CTGGCAGGAGATTCCAAGGAT<br>RV-TCTGCCAGTGCCTCTTTGC | 167 | NM_001003301 |
| *IL-8* | FW-TTGCTCTCTTGGCAGCTTTTG<br>RV-TTTGGGATGGAAAGGTGTGG | 122 | NM_001003200 |
| *IL-10* | FW-ACATCAAGAACCACGTGAACTCC<br>RV-ACTCACTCATGGCTTTGTAGACACC | 177 | NM_001003077 |

**Table 10 (continue) - Primers sequences used for quantitative real time PCR (qRT-PCR)**

| **Target Gene** | **Sequence** (5′-3′) | **Product size** (bp) | **GeneBank accession number** |
|---|---|---|---|
| *TGF-β1* | FW-AGCCCGAGGCGGACTACTAC<br>RV-CGGAGCTCTGATGTGTTGAAGA | 130 | NM_001003309 |
| *IFN-β* | FW – GAAGCTCCACTGGCAGAAGG<br>RW- TGGCCTTCAGGTACTGCACA | 137 | NM_001135787.1 |
| *CXCL10* | FW- TCCTGCAAGTCCATCGTGTC<br>RV-ATTGCTTTCACTAAACTCTTGATGGTC | 114 | NM_001010949.1 |
| *RPL27* | FW-TCGTCAACAAGGATGTCTTCAGAG<br>RV-TCTTGCCAGTCTTGTACCTCTCCT | 96 | NM_001003102 |

### 3.2.5 Immunofluorescence

After the stimulation assay, epithelial and stromal cells were washed three times in sterile PBS, fixed for 10min in 4% paraformaldehyde (Sigma), washed two times in PBS- 0.1% Triton for 5 min and incubated with the blocking solution (PBS + 2.5% BSA) for 1h at room temperature. Cells were incubated overnight at 4ºC with rabbit polyclonal anti-IRF3 antibody (1:50, ab25950, Abcam®, Cambridge, UK) and rabbit polyclonal anti-NFkB p65 antibody (1:100, sc-109-G, Santa Cruz biotechnology®, Texas, USA). After washing twice for 10 min in PBS, the secondary antibody (1:300, Alex Fluor® 594 Goat Anti-Rabbit IgG (H+L), Life Technologies, Carlsbad, USA) was added and cells incubated for 1h at room temperature. After washing for 10 min in PBS, the glass coverslips were mounted in Vectashield® mounting medium with DAPI (H-1200, Vector Laboratories Inc., Burlingame, CA, USA). Slides were observed using a fluorescence microscope (Leica DM5000B) and the images obtained in Adobe Photoshop CS5.

### 3.2.6 Statistical analysis

Data were analyzed through a statistical software package (Statistica 5.0, StatSoft Inc., Tulsa, OK, USA, 1995). Categorical data was analyzed by Fisher's exact test. Hemogram,

cell number and cytokine transcription data were log-transformed (log x + 1) to normalize distribution. Hemogram, blood biochemical and endometrial cytokine transcription data (Experiment 1) and cell number (Experiment 2) were analyzed by ANOVA. In stromal cells Pyo18 induced cell death after 4h of incubation, so transcription data was not available. Therefore cell cytokine transcription data (Experiment 2) were analyzed using the MANOVA factorial procedures with three fixed effects: type of cells (n = 2; stromal cells, epithelial cells); type of stimulus (n = 4; LPS, Pyo14, Pyo18*ΔhlyA*); time of incubation (1h, 4h) and their interactions. Additionally, a MANOVA factorial procedure with two fixed effects (type of cells and type of stimulus, including Pyo18) was used to analyze data at 1h of incubation. Significant effects were further analyzed and means compared using the Scheffé test. Significance was determined at the 5% confidence level ($p < 0.05$).

## 4. Results

### 4.1. Hemolytic *E. coli* induces a more extensive uterine damage and inflammatory cell infiltration than non-hemolytic *E. coli* (Experiment 1)

Hemolytic and non-hemolytic *E. coli* pyometra bitches showed similar hemogram and biochemical blood results (Table 11). All pyometra uterine samples showed destroyed luminal epithelium, damaged apical glands, inflammatory cellular infiltrate in the stromal and glandular compartments and cystic endometrial glands. Interstitial edema, blood extravasation and destroyed basal glands were observed in 52-68% of cases. However, 6 of 8 (75%) of hemolytic *E. coli* pyometra samples had histological evidence of metritis, whereas this was only observed in 2 of 10 (20%) of non-hemolytic *E. coli* pyometra cases ($p< 0.05$) (Figure 14A).

An extensive stromal infiltration of myeloid cells (granulocytes/macrophages) (Figure 14B b, c) and B lymphocytes was observed, being B lymphocytes more predominant in the apical than in the basal layer (Figure 14B e, f). Stromal infiltration of T lymphocytes was moderate and more predominant in the apical layer (Figure 14B h, i). Most (84%) of the pyometra samples also showed an extensive infiltration of myeloid cells in endometrial glands, whereas the glandular infiltration of lymphocytes was absent or weak.

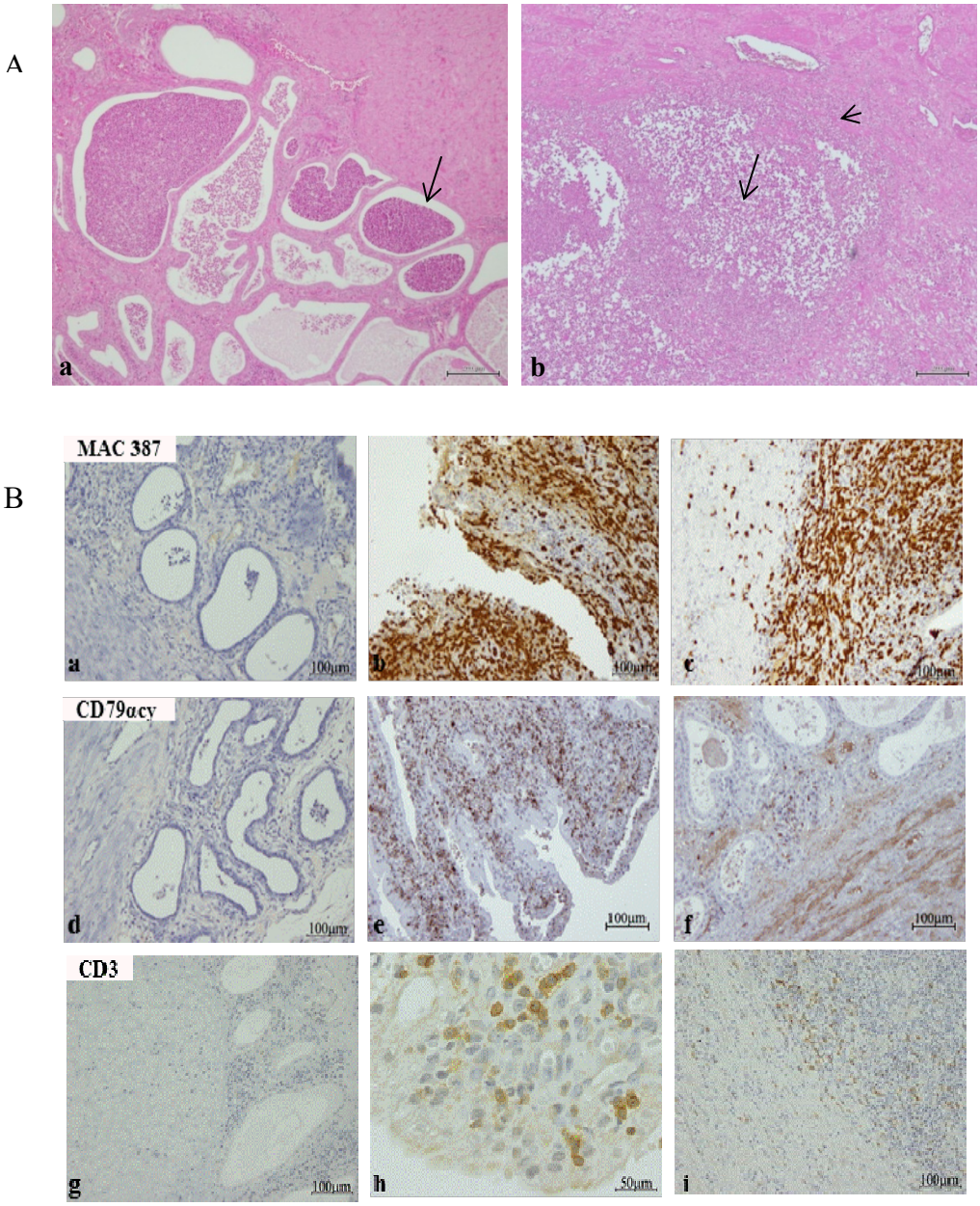
The presence of inflammatory cells in normal diestrous uteri was scarce (detected in 30% of the samples) and consisted of few granulocytes/macrophages (apical and basal layer) and T cells (apical layer) in the stromal compartment.

**Table 11 - Haematological and biochemical parameters in bitches with hemolytic *E. coli* pyometra (n=8) and with non-hemolytic *E. coli* pyometra (n=10)**

| **Haematological parameters** | **Hemolytic *E. coli* pyometra** (Mean ± SEM) | **Non-hemolytic *E. coli* pyometra** (Mean ± SEM) |
|---|---|---|
| White blood cell count (x $10^3$/µl) | 45,1 ± 5,5 | 33,8 ± 9,1 |
| Eritrocytes (x $10^6$/µl) | 5,5 ± 0,1 | 6,1 ± 0,4 |
| Platelet Count (x $10^3$/µl) | 216,1 ± 66,3 | 247,4 ± 48,7 |
| Hemoglobin (g/dL) | 12,6 ± 0,3 | 13,7 ± 0,8 |
| Hematocrit (%) | 37,3 ± 0,9 | 40,2 ± 2,4 |
| Mean cell volume (fl) | 67,6 ± 0,9 | 66,1 ± 1,0 |
| Mean cell hemoglobin (pg) | 22,9 ± 0,4 | 22,6 ± 0,2 |
| Mean cell hemoglobina concentration (g/dl) | 33,9 ± 0,6 | 34,2 ± 0,4 |
| Neutrophils (/µl) | 34750,5 ± 4239,2 | 25786,0 ± 7279,2 |
| Band Neutrophils (/µl) | 938,3 ± 714,4 | 2216,7 ± 1092,7 |
| Lymphocytes (/µl) | 3449,2 ± 574,6 | 2317,3 ± 373,0 |
| Monocytes (/µl) | 5576,8 ± 1808,6 | 3208,6 ± 922,9 |
| Eosinophils (/µl) | 238,8 ± 122,1 | 304,8 ± 207,6 |
| Basophils (/µl) | 275 ± 275 | 0 |
| **Bioquimical parameters** | | |
| Alanina-aminotransferase (U/L 25ºC) | 22,5 ± 3,9 | 38,1 ± 12,5 |
| Alkaline phosphatase (U/L) | 309,2 ± 146,5 | 488,1 ± 311,8 |
| Creatinine (mg/dl) | 1,3 ± 0,2 | 1,1 ± 0,1 |
| Urea (mg/dl) | 30,6 ± 9,7 | 38,3 ± 17,2 |

For all parameters p> 0.05

**Figure 14 - Histological section of uterine pyometra samples (A) and immunostaining of calprotectin positive cells (myeloid cells), CD79 αcy positive cells (B lymphocytes) and CD3 positive cells (T lymphocytes) in pyometra uterine sections (B)**




(A) Histological section of uterine pyometra samples: a - non-hemolytic *E. coli* pyometra evidencing basal glands with myeloid cells infiltration (arrow); b – β-hemolytic *E. coli* pyometra evidencing extensive damage of basal glands (arrow) and metritis (arrow head). Staining by H&E. (B) Immunostaining of calprotectin positive cells (myeloid cells), CD79 αcy positive cells (B lymphocytes) and CD3 positive cells (T lymphocytes) in pyometra uterine sections. Myeloid cells (granulocytes /macrophages) detection: a- negative control (staining with mouse isotype); b,c- infiltration in apical and basal layer, respectively (cytoplasmic staining pattern). B lymphocytes detection: d- negative control (PBS); e,f- infiltration in apical and basal layer, respectively (membrane staining pattern). T lymphocytes detection: g- negative control (staining with rabbit isotype); h,i- infiltration in apical and basal layer (membrane and cytoplasmic staining pattern), respectively.

### 4.2. Hemolytic and non-hemolytic *E. coli* pyometra endometria have different transcription levels of genes coding pro-inflammatory cytokines (Experiment 1)

Constitutive transcription of MyD88-dependent (MyD88, TRAF6, NFKβ) and independent (TRAM, TRIF, IRF3) pathway components was detected in healthy diestrous and pyometra endometria (Figure 15A). *IL-2*, *IL-4*, *IFNβ* and *IFNγ* transcripts were not detected in both endometria types. Therefore, *MyD88*, *TRAM*, *TRAF6*, *TRIF*, *NFκB*, *IRF3*, *IL-2*, *IL-4*, *IFNβ* and *IFNγ* genes were not analysed by qRT-PCR.

Overall, transcription levels of pro-inflammatory cytokines *IL-1β*, *IL-6* and *IL-8*, and of anti-inflammatory cytokines *IL-10* and *TGFβ* were significantly higher ($p < 0.0001$) in pyometra endometria than in healthy diestrous endometria. However, hemolytic *E. coli* pyometra endometria had higher transcription levels of *IL-1β* and *IL-8* and lower transcription levels of *IL-6* than non-hemolytic *E. coli* endometria ($p < 0.01$) (Figure 15B).

**Figure 15 – Representative PCR detection of transcripts of TLRs signaling components and of cytokines in diestrous and pyometra endometria (A). Relative mRNA expression level of *IL-1β*, *IL-6*, *IL-8*, *IL-10*, and *TGFβ* evaluated by real-time PCR (B)**

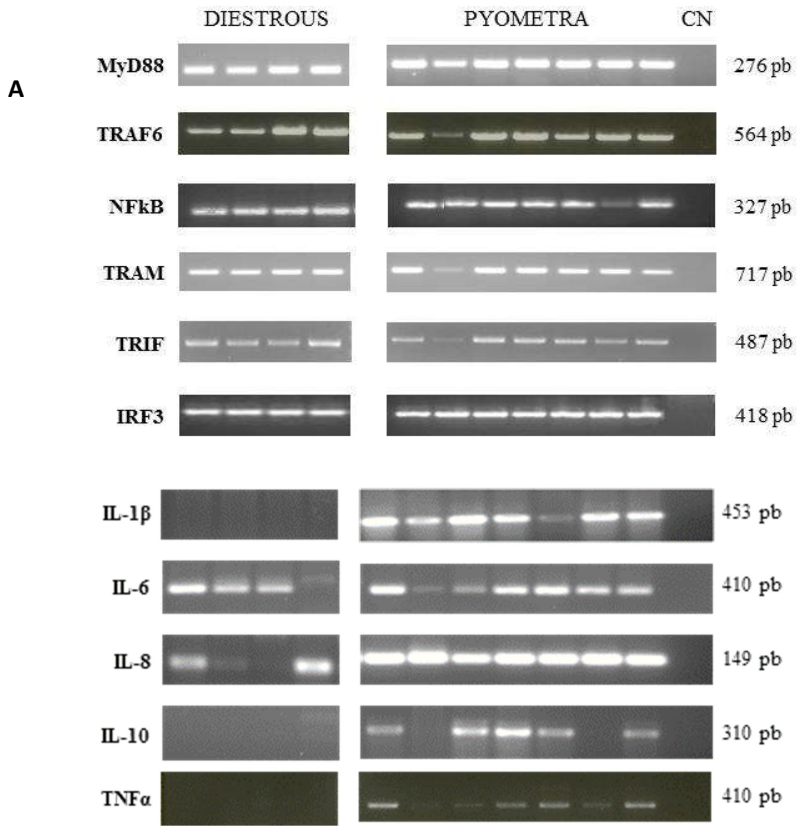
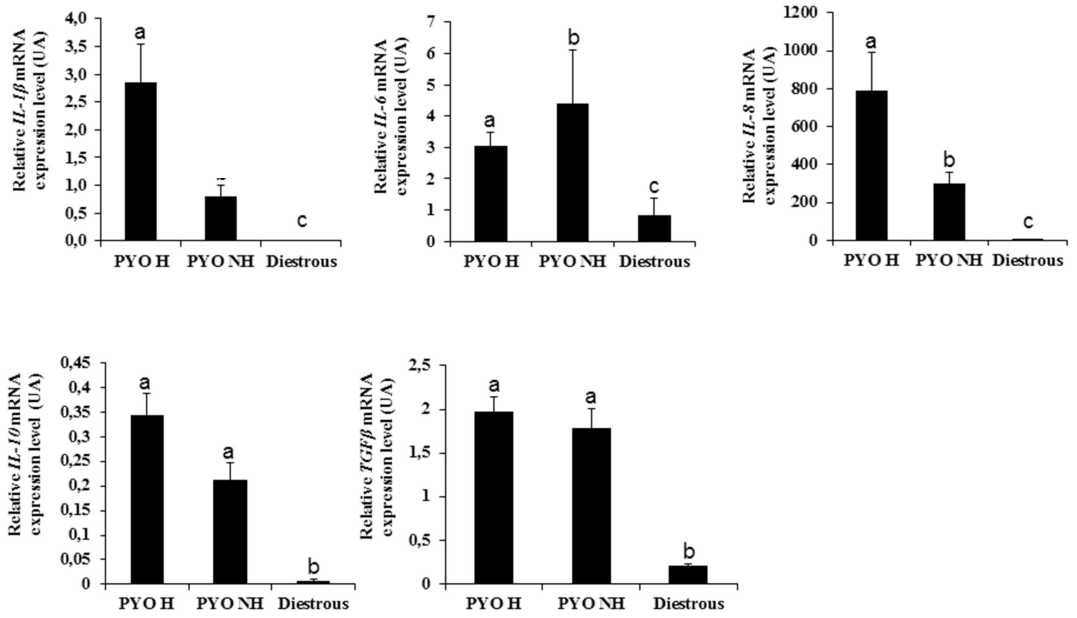


**Figure 15 (continuation) – Representative PCR detection of transcripts of TLRs signaling components and of cytokines in diestrous and pyometra endometria (A). Relative mRNA expression level of *IL-1β*, *IL-6*, *IL-8*, *IL-10*, and *TGFβ* evaluated by real-time PCR (B)**

B




(A) Representative PCR detection of transcripts of TLRs signaling components and of cytokines in diestrous and pyometra endometria. NC - negative control. (B) Relative mRNA expression level (arbitrary units, AU) of *IL-1β*, *IL-6*, *IL-8*, *IL-10*, and *TGFβ* evaluated by real-time PCR. Data are given as mean ± SEM. Columns with different superscripts differ significantly (*IL-1β*, *IL-6*, *IL-8*: $^{ab}$ $p<0.01$ $^{ac/bc}$; $p<0.00001$; *IL-10*, *TGFβ*: $^{ab}$ $p<0.0001$). MyD88 (myeloid differentiation factor 88); TRAM (TRIF related adaptor molecule); TRAF6 (TNFR-associated factor 6); TRIF (TIR domain-containing adaptor inducing interferon (IFN)-β); IRF3 (interferon regulatory factor 3).

## 4.3 Cytotoxicity due to α-hemolysin is mainly targeted to stromal endometrial cells (Experiment 2)

In epithelial cells, bacterial adhesion after 1 and 4h of incubation was similar (3-5%) for both *E. coli* strains. This low bacterial adhesion was also observed in stromal cells incubated with Pyo14. In both epithelial and stromal cells incubated with Pyo14, bacterial internalization was absent (at 1h) or very low (at 4h; 0.1 – 0.2%). Incubation of stromal

cells with Pyo18 for 4h resulted in the death of most cells, therefore bacterial adhesion and internalization could not be evaluated.

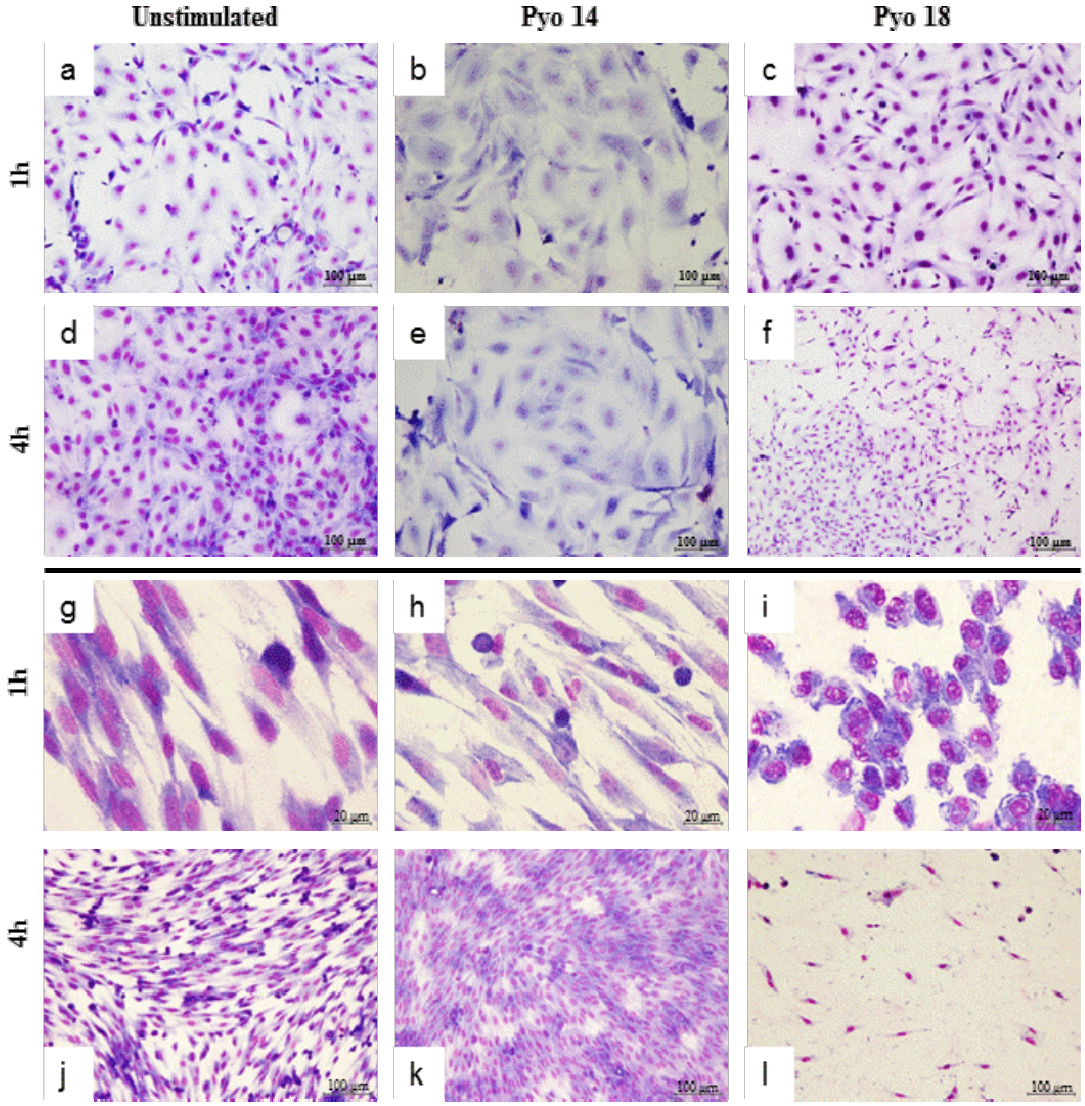
**Table 12 - Effect of bacterial and LPS incubation on endometrial epithelial and stromal cell numbers.**

| | Stromal Cells | | Epithelial Cells | |
|---|---|---|---|---|
| ***E. coli* strain** | 1H | 4H | 1H | 4H |
| Pyo14 | 102.7 ± 5.9 | 98.9 ± 2.5 * | 107.0 ± 1.4 | 96.3 ± 6.3* |
| Pyo18 | 102.9 ± 4.8 [a] | 0 [b] ** | 105.9 ± 3.3 [c] | 54.3 ± 3.4 [d]** |
| Pyo18*ΔhlyA* | 104.5 ± 3.0 | 97.4 ± 3.2 * | 110.1 ± 2.3 | 97.9 ± 4.4* |
| LPS | 100.7 ± 8.4 | 96.0 ± 4.6 * | 115.8 ± 2.2 | 99.9 ± 6.4* |

[ab] $p < 0.00001$; [cd] $p < 0.0001$; [*/**] $p < 0.01$

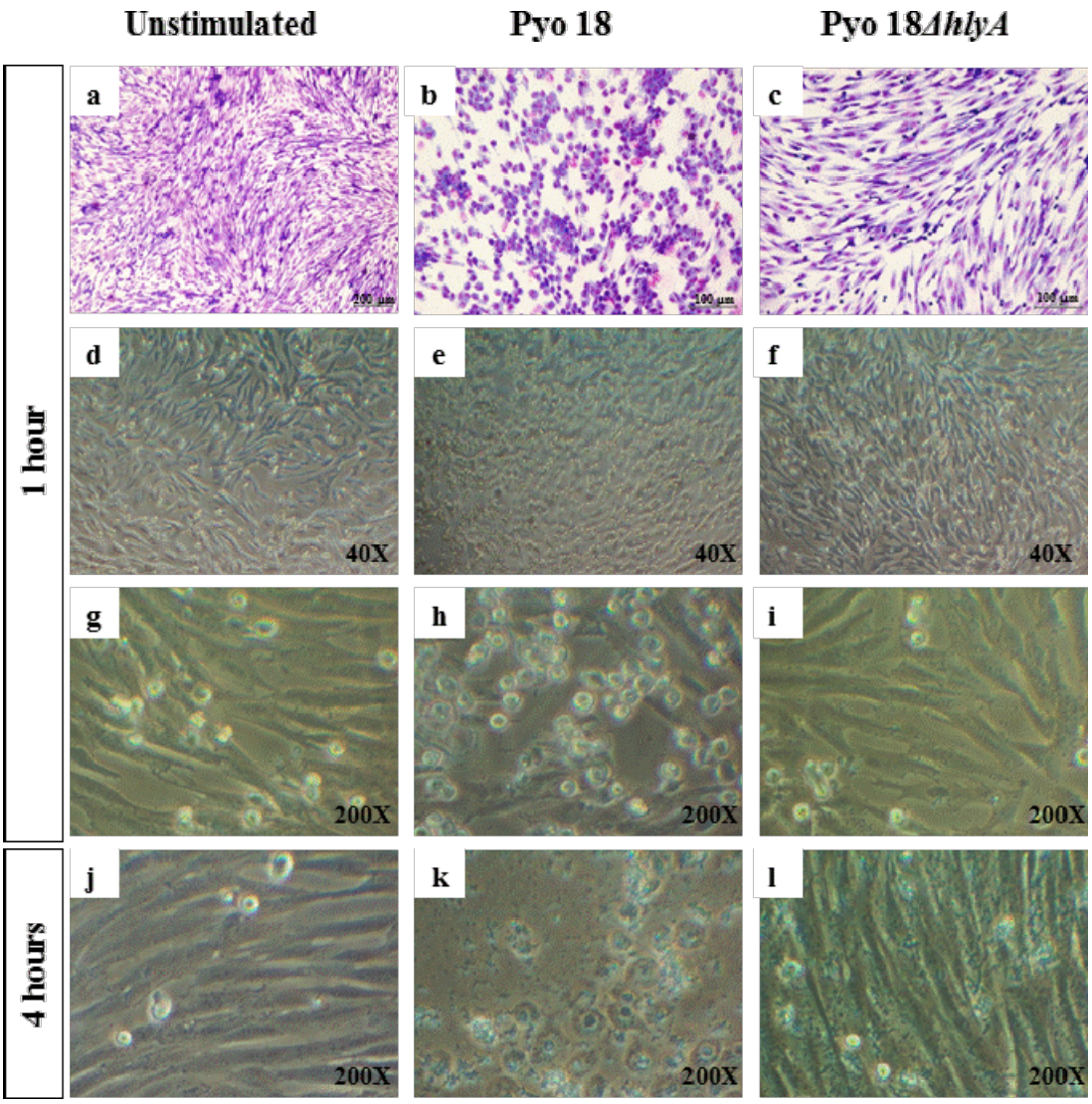
Incubation with Pyo14 did not affect cell numbers and cell morphology in both types of cells (Table 12; Figure 16). Incubation with Pyo18 induced a decrease in epithelial cell numbers at 4h ($p < 0.001$; Table 1), although the morphology of surviving cells was not affected (Figure 3 c, f). Incubation of stromal cells with Pyo18 for 1h did not affect cell numbers (Table 12), but cells lost the typical elongated fibroblast shape, becoming spherical (Figure 16 I; Figure 17 b, e, h). As stated above, incubation of stromal cells for 4h with Pyo18 induced the detachment and death of all the cells (Figure 16; Figure 17 k).
In contrast, incubation with the isogenic mutant Pyo18*ΔhlyA* did not induce a cytotoxic effect on stromal cells (Figure 17 c, f, i, l). In fact, following incubation with this mutant strain, cell morphology and numbers were similar to those observed after incubation with Pyo14 (Table 12, Figure 17).

**Figure 16 - Morphology of endometrial epithelial (a-f) and stromal (g-l) cell cultures stained with Giemsa after 1 and 4 h of incubation.**




Cell cultures stained with Giemsa after 1 and 4 h of incubation: unstimulated cells (a, d g, j); endometrial epithelial (b, e) and stromal (h,k) cells incubated with Pyo14 and endometrial epithelial (c,f) and stromal (i,l) cells incubated with Pyo18.

**Figure 17 - Morphology of endometrial stromal cells after 1 and 4 h of incubation, evaluated after Giemsa staining (a-c) or under phase-contrast (d-l).**




Morphology of endometrial stromal cells after 1 and 4 h of incubation, evaluated after Giemsa staining or under phase-contrast: unstimulated cells (a, d, g, j); cells incubated with Pyo18 evaluated after Giemsa staining (b) or under phase-contrast (e,h,k); cells incubated with Pyo18*ΔhlyA* evaluated after Giemsa staining (c) or under phase-contrast (f,i,l)

### 4.4 Hemolytic and non-hemolytic *E. coli* strains induce differential immune response in endometrial epithelial and stromal cells

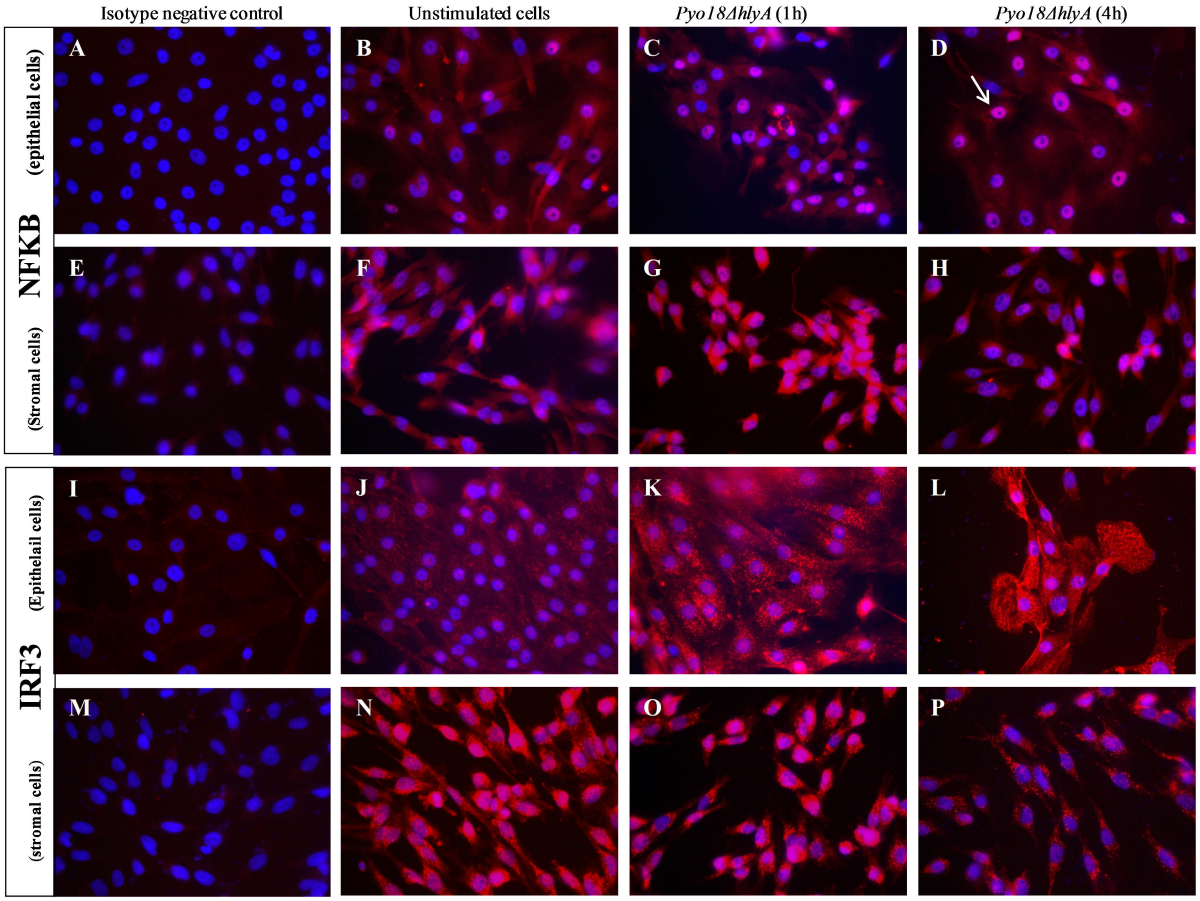
Transcription of *IRF3*, *IRF7* and *NFkB* genes was not affected by type of cells, type of bacteria or incubation time (data not shown). Translocation of IRF3 and NFkB to the nucleus was detected by IHC (Figure 18) in endometrial epithelial and stromal cells.

In epithelial cells, Pyo18, Pyo18*ΔhlyA* and Pyo14 induced similar transcription levels of cytokines in each endpoint of incubation (Figure 19). Stimulation with Pyo18, induced a higher *IL-6* (1h vs 4h: 6.0 vs 24.9 fold increase, $p< 0.01$) (Figure 19C) mRNA expression after 4h than after 1h of incubation. All bacterial stimuli induced a significant increase in transcription levels of *CXCL10* after 4h (1h vs 4h: Pyo18 – 19.2 vs 105.2; Pyo14 – 14.9 vs 146.5; Pyo18*ΔhlyA* - 19.3 vs 142.4; LPS - 17.8 vs 126.3 fold increase, $p< 0.01$) (Figure 19F), and a decrease of *IFNβ* after 4h (1h vs 4h: Pyo18 - 137.4 vs 7.8; Pyo14 - 75.5 vs 4.0; Pyo18*ΔhlyA* - 108.2 vs 24.1; LPS – 62.0 vs 2.5 fold increase, $p< 0.001$) (Figure 19H).

In stromal cells, after 1h of incubation and compared to the isogenic mutant Pyo18*ΔhlyA* and to Pyo14, respectively, Pyo18 induced a lower transcription level of *IL-1β* (0.99 vs 152.0 vs 50.9 fold increase, $p< 0.001$) (Figure 19A), *TNFα* (3.2 vs 49.9 vs 12.9 fold increases, $p< 0.05$) (Figure 19D) and *IL-10* (0.4 vs 3.6 vs 2.6 fold increases, $p< 0.001$) (Figure 6G). Stimulation with Pyo14, induced higher *IL-1α* and *IL-8* transcription levels after 4h than 1h of incubation (1h - 2.2 vs 4h - 7.9 fold increase, $p< 0.05$ and 1h - 19.8 vs 4h – 184.1 fold increase, $p< 0.01$, respectively) (Figure 19B, 19E). Stimulation with Pyo18*ΔhlyA* also induced higher *IL-1α* and *IL-8* transcription levels after 4h than 1h of incubation (1h - 4.5 vs. 4h - 9.9 fold increase, $p< 0.05$ and 1h - 4.5 vs. 4h - 168.7 fold increase, $p< 0.01$, respectively) (Figure 19B, 19E). Transcription of *INFβ* was not detected in stromal cells.

The bacterial stimuli induced different gene transcription levels in endometrial epithelial and stromal cells. Pyo14 and Pyo18*ΔhlyA* induced higher transcription levels of *CXCL10* after 4h of incubation in epithelial than in stromal cells (Pyo14 - 146.5 vs 58.9 fold increase, $p< 0.05$ and Pyo18*Δhly* - 142.4 vs 44.9 fold increase, $p< 0.01$, respectively) (Figure 19F). Also, Pyo14 and Pyo18*ΔhlyA* induced higher transcription levels of *IL-8* in stromal than epithelial cells after 4h of incubation (Pyo14 - 184.7 vs 6.7 fold increase; Pyo18*ΔhlyA* - 168.7 vs 19.8 fold increase, respectively, $p< 0.02$) (Figure 19E).

**Figure 18 - Nuclear detection of NFκB (A-H) and IRF3 (I-P) in endometrial epithelial and stromal cells by immunofluorescence**.




A-D and I-L- epithelial cells [A,I - rabbit isotype negative control after 4h of incubation; B,J- unstimulated cells after 4h of incubation; C,D and K,L- stimulation with *Pyo18ΔhlyA* strain during 1h (C,K) and 4h (D,L)]; E-H and M-P- stromal cells [E,M- rabbit isotype negative control after 1h of incubation; F,N-unstimulated cells after 1h of incubation; G,H and O,P- stimulation with *Pyo18ΔhlyA* strain during 1h (G,O) and 4h (H,P)]. White arrow – nuclear staining.

**Figure 19 - *Escherichia coli* or LPS-induced cytokines gene transcription in cultured canine endometrial epithelial and stromal cells: *IL-1β* (A), *IL-1α* (B), *IL-6* (C), *TNFα* (D), *IL-8* (E), *CXCL10* (F), *IL-10* (G), *INFβ* (H) in cultured canine endometrial epithelial and stromal cells in response to hemolytic *E. coli* (Pyo18), non-hemolytic *E. coli* (Pyo14) and the isogenic mutant of Pyo18 (Pyo18*ΔhlyA)*.**

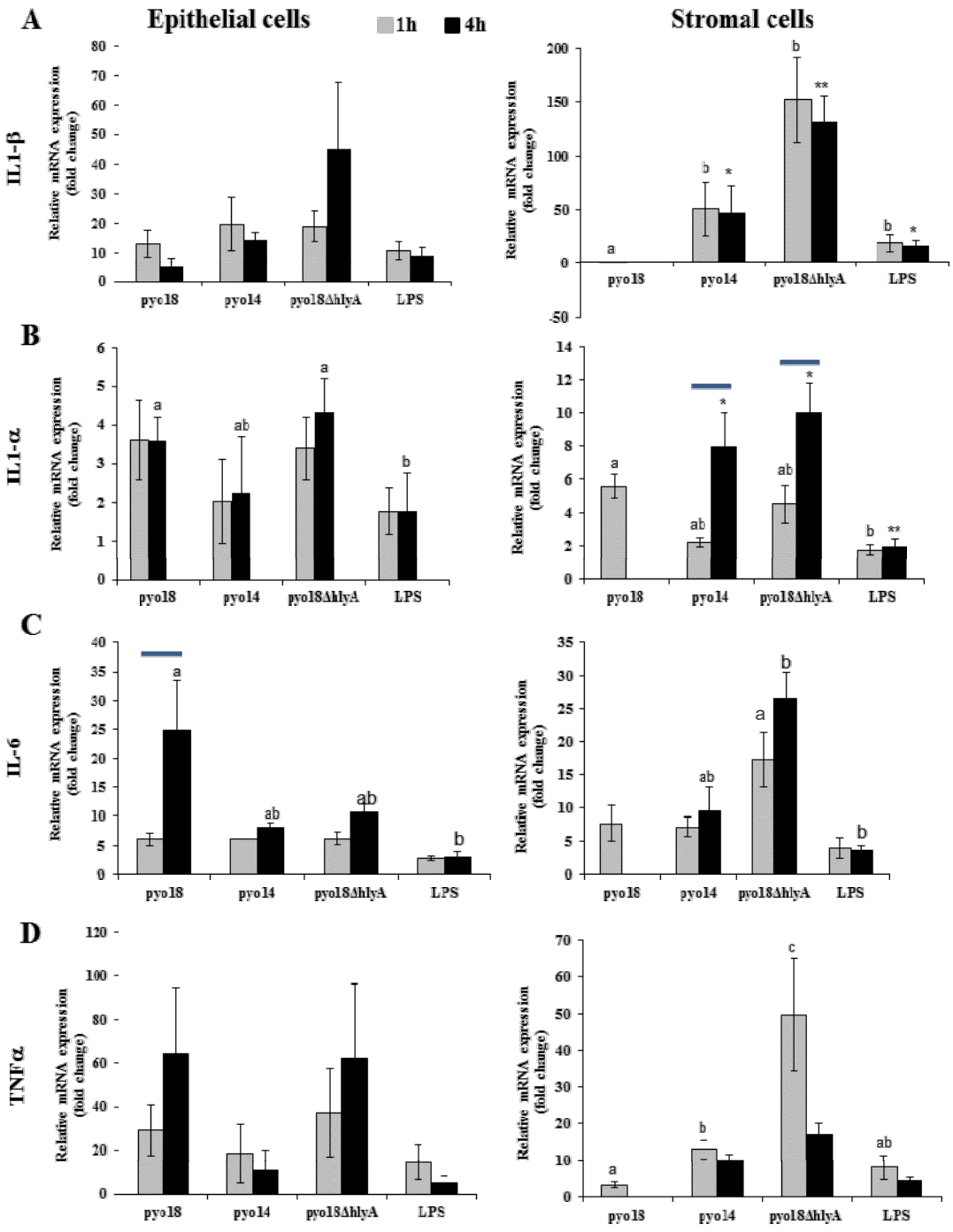
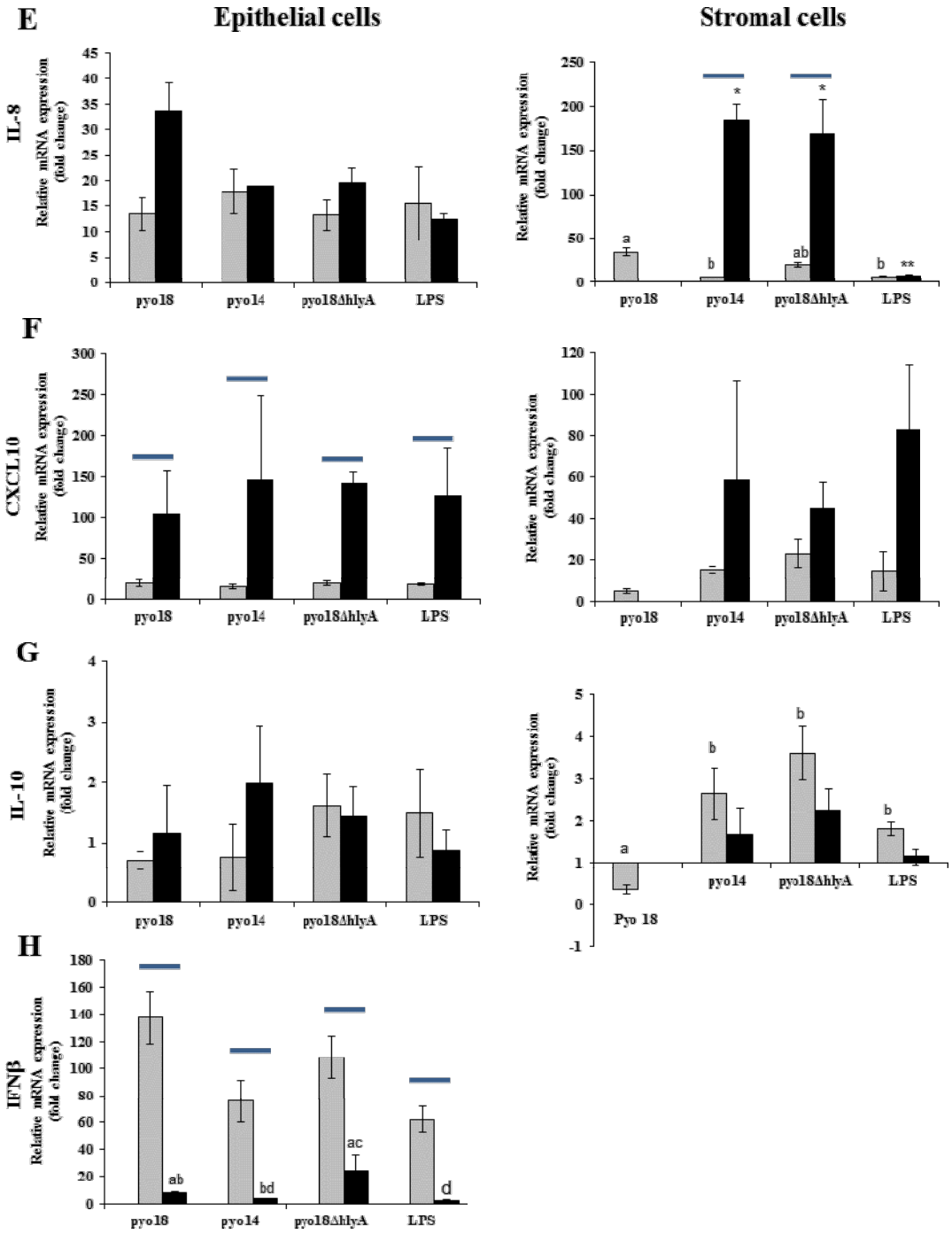


**Figure 19 (continuation) - *Escherichia coli* or LPS-induced cytokines gene transcription in cultured canine endometrial epithelial and stromal cells: *IL-1β* (A), *IL-1α* (B), *IL-6* (C), *TNFα* (D), *IL-8* (E), *CXCL10* (F), *IL-10* (G), *INFβ* (H) in cultured canine endometrial epithelial and stromal cells in response to hemolytic *E. coli* (Pyo18), non-hemolytic *E. coli* (Pyo14) and the isogenic mutant of Pyo18 (Pyo18*ΔhlyA)*.**



Relative mRNA expression of *IL-1β* (A), *IL-1α* (B), *IL-6* (C), *TNFα* (D), *IL-8* (E), *CXCL10* (F), *IL-10* (G), *INFβ* (H) in cultured canine endometrial epithelial and stromal cells in response to hemolytic *E. coli* (Pyo18), non-hemolytic *E. coli* (Pyo14) and the isogenic mutant of Pyo18 (*Pyo18ΔhlyA).* Treatment with 1μg/ml of LPS was used as a positive control. Data are mean ± S.E.M. Transcription levels (fold increase) are normalized to those of non-stimulated cells. $^{ab}$ $p < 0.05$ [(Epithelia cells: *IL-1α*, *IL-6*, *IFNβ*; stromal cells: *IL-1β*, *IL-1α*, *IL-8*, *IL-10*]; $^{ab}$ $p < 0.01$ (stromal cells: *IL-6*, *IL-10*); */**$p < 0.05$ (*IL-1β*); */**$p < 0.01$ (*IL-1α*, *IL-8*); $p< 0.05$ (1h vs 4h: *IL-1α*); $p< 0.01$ (1h vs 4h: *IL-6*, *IL-8*, *CXCL10*); $p< 0.001$ (1h vs 4 h: *IFNβ*).

## 5. Discussion

*Escherichia coli* is isolated from the uterus of up to 90% of bitches with pyometra (Dhaliwal et al., 1998; Mateus et al., 2013), and β-hemolytic *E. coli* is found in 35-52% of *E. coli* pyometra cases (Chen et al., 2003; Mateus et al., 2013; Siqueira et al., 2009). Although this latter prevalence leads to the suggestion that α-hemolysin contributes to the virulence of *E. coli* strains, the role of this toxin in the pathogenesis of canine pyometra is unknown. The results of this study indicate that α-hemolysin contributes to the virulence of *E. coli*, by inducing tissue damage and a compromised early uterine immune response. On one side, the cytotoxic activity is mainly directed towards stromal cells, facilitating progression of bacteria into the uterine tissue. On the other side, the modulation of the uterine innate immune response may induce a delayed neutrophil influx to the uterus. Additionally, the known cytotoxic effect of hemolysin in granulocyte and lymphocytes as well as the enhancement of access to cell nutrients and iron storages (reviewed by Bien et al., 2012) might also enhance hemolytic *E. coli* survival within the uterine tissue.

The relationship between the isolation of β-hemolytic *E. coli* and clinical signs and histopathological findings was not reported. UPEC strains that express HlyA were shown to cause more extensive tissue damage within the urinary tract, which was correlated with more severe clinical outcomes (Smith et al., 2008). In this study, clinical signs, hematological and blood biochemical results were similar in hemolytic and non-hemolytic *E. coli* pyometra bitches. However, β-hemolytic *E. coli* was associated with high endometrial damage and metritis. Bacteria and endotoxins are potent inducers of an inflammatory response. All *E. coli* pyometra uteri evidenced an infiltrate of inflammatory cells, including neutrophils, lymphocytes and macrophages. This induces the up-regulation of TLR2 and TLR4 transcription (Hagman et al., 2009; Silva et al., 2010) and expression (Silva et al., 2012) and

the consequent up-regulation of gene transcription of several pro- and anti-inflammatory cytokines, including *IL-1β*, *IL-6*, *IL-8*, *IL-10* and *TGFβ*. However, hemolytic *E. coli* endometria had higher transcription levels of *IL-1β* and *IL-8* and lower transcription levels of *IL-6* than non-hemolytic *E. coli* endometria. IL-6 stimulates leukocyte activation and myeloid progenitor cell proliferation, regulates the synthesis of acute phase proteins and is a powerful pyrogen (Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011). In pyometra cases, C-reactive protein, serum amyloid A and haptoglobin concentrations can be used to monitor early post-OVX complications and ongoing inflammation (Dabrowski, Kostro, Lisiecka, Szczubiał, & Krakowski, 2009). C-reactive protein was also associated with SIRS (Fransson et al., 2007). High serum levels of IL-8 were observed in pyometra bitches, especially in those that developed SIRS, suggesting IL-8 as a useful early biomarker of uterine infection and, possibly, of sepsis (Karlsson et al., 2012). IL-8 is also a potent neutrophil chemotactic molecule. Neuthropil recruitment to the site of infection was shown to be critical for bacterial clearance in the uterus. However, their action may also lead to tissue damage.

Endometrial epithelial cells showed a high sensitivity to the cytotoxic effect of HlyA. Following 4h of incubation with Pyo18, epithelial cell numbers decreased by 46%. This was not observed following the incubation with Pyo14 and Pyo18*ΔhlyA*, where cell numbers and viability were not affected after 4h of incubation. This may lead to the suggestion that β-hemolytic *E. coli* induces an earlier damage to the epithelial layer than non-hemolytic *E. coli* strains. During uterine contamination, epithelial cell apoptosis (Smith, Grande, Rasmussen, & O'Brien, 2006) and/or degradation of proteins involved in cell-cell and cell-matrix interactions (Dhakal & Mulvey, 2012) due to the sublytic concentrations of HlyA may be the reason for the disruption of the epithelial layer due to β-hemolytic *E. coli*. Endometrial stromal cells showed a higher sensitivity to the cytotoxic effect of HlyA than epithelial cells. Following 4h of incubation with Pyo18, no stromal cells survived. Again, this was not observed following the incubation with Pyo14 and Pyo18*ΔhlyA*. A higher sensitivity of bovine stromal cells to pyolysin-mediated cytolysis of *Trueperella pyogenes* was recently reported (Amos et al., 2014). This sensitivity of both epithelial and stromal cells to the cytotoxic effect of HlyA may explain the observed significant association between β-hemolytic *E. coli* isolation and metritis.

Adhesion is the first step of infection and adhesion of *E. coli* to canine endometrium is enhanced during early diestrus (Ishiguro et al., 2007). Adhesion to epithelial and stromal cells

was similar following bacterial incubation, irrespective of *E. coli* strain. The observed level of *E. coli* adhesion is similar to that reported for bovine endometrial cells (Sheldon et al., 2010). Pyo14 and Pyo18 have genes for Type 1 fimbrae and S + F1C fimbriae, whereas *papG* gene that codes for P fimbriated was only detected in Pyo18 (Henriques et al., 2014). Although adherence of *E. coli* to canine endometrial cells was shown to be mediated by Type 1 fimbriae (Krekeler et al., 2012), the targeted deletion of specific adhesin genes in a canine pyometra strain was compensated by the presence of other adhesins, which indicates functional redundancy among adhesins (Krekeler et al., 2013).

Bacterial internalization was absent or very low. This indicates that cell invasion is not necessary for inducing the cytotoxic effect of HlyA. Poor internalization of hemolytic *E. coli* was reported in HeLa and HTB-4 cell co-culture systems (Saldaña et al., 2014). Low internalization was also observed in bovine endometrial epithelial and stromal cells following incubation with *E. coli* during 4h (Sheldon et al., 2010).

Canine endometrial epithelial and stromal cells express TLR4 (Silva et al., 2012), a receptor that is activated by lipid A of LPS, and by *E. coli* Type 1 fimbriae (through the MyD88 dependent pathway) and P fimbriated (through the TRAM /TRIF pathway) (Fischer, Yamamoto, Akira, Beutler, & Svanborg, 2006). TLR4 binding triggers a signaling cascade that culminates in the activation of NFkB and IRF3. Upon activation, NF-κB and IRF3 translocate to the nucleus and induce the transcription of target genes, including those coding for acute phase proteins and pro-inflammatory cytokines and chemokines, and those coding for interferons, respectively. This signaling contributes to the recruitment of inflammatory cells to the site of infection (Horne et al., 2008). In this study, the detection of NFkB and IRF3 in the nucleus, together with the observed cytokine transcription results, indicates that the TLR signaling pathway was activated.

The transcription of *CXCL10* was significantly higher in endometrial epithelial than stromal cells. A high transcription level of *CXCL10* was also observed in canine endometrial stromal cells after stimulation with *E. coli* and LPS (Karlsson et al., 2015). CXCL10 displays antimicrobial activity against *E. coli* (Cole et al., 2001), and likewise defensins, may act as the first line of defense in epithelia (Gallo, Murakami, Ohtake, & Zaiou, 2002). CXCL10 is also involved in the recruitment and potentiation of T-helper 1 response (Dufour et al., 2002). However, $\gamma\delta^{+}$/CD8$^{-}$ T lymphocytes were the only significant lymphocyte population detected in pyometra uteri (Bartoskova et al., 2012).

In contrast to *CXCL10*, *IL-8* had a higher transcription level in stromal than in epithelial cells after 4h incubation with Pyo14 and Pyo18*ΔhlyA*. A high transcription level of *IL-8* in canine stromal cells after *E. coli* and LPS stimulation was reported by Karlsson et al. (Karlsson et al., 2015). Stromal cells also had a high level of *IL-1β* mRNA expression. This may indicate that, during uterine *E. coli* infection and after disruption of the epithelial layer of the endometrium, stromal cells are an important early source of IL-1β and IL-8, major cytokines involved in the recruitment of neutrophils and macrophages. Interestingly, Pyo18 decreased the transcription level of *IL-1β* in stromal cells. Recently, in a bacteremia mouse model, α-hemolysin inhibited IL-1β secretion (Diabate et al., 2015).

Pyo18, Pyo18*ΔhlyA* and Pyo14 induced *IL-6* gene transcription in endometrial epithelial and stromal cells. In the presence of a confluent epithelium, IL-6 is secreted apically by the polarized epithelial cells (Healy, Cronin & Sheldon, 2015). Therefore, unless the epithelium is breached, immune cells may only be exposed to IL-6 once they reach the lumen. Pyo18*ΔhlyA* and Pyo14 induced an increase in *IL-1α* transcription in endometrial stromal cells that was significantly higher than after LPS stimulation. IL-1α is produced following a PAMP stimulus, but is retained intracellular in undamaged cells (Healy, Cronin & Sheldon, 2014). If there is a combination of pathogen challenge followed by cell damage, IL-1α acts to scale the endometrial cell inflammatory response (Healy et al., 2014).

Following bacterial stimulation, endometrial epithelial and stromal cells evidenced an upregulation of *TNFα* transcription. However, *TNFα* transcription levels in stromal cells were lower following Pyo18 stimulation than following Pyo18*ΔhlyA* and Pyo14 stimulation. The decrease in *TNFα* is probably responsible for the low levels of *IL-1β* also observed in stromal cells after Pyo18 incubation. König & König (König & König, 1993) reported a down-regulation of TNFα expression in human inflammatory cells stimulated with hemolytic *E. coli*. In contrast, a low level of *TNFα* transcription was observed in pyometra endometria (Experiment 1). This is in accordance with the findings of Hagman et al. (Hagman et al., 2009), and may reflect the late diagnosis of the disease. Also, serum TNFα concentrations were similar in dogs with or without pyometra (Karlsson et al., 2012).

Pyometra uteri (Experiment 1) and endometrial epithelial and stromal cells (Experiment 2) evidenced an up-regulation of anti-inflammatory cytokine *IL-10* mRNA expression. IL-10 inhibits the release of TNFα, IL-1β, IL-6 and IL-12 from monocytes and macrophages, and induces the production of IL-1 receptor antagonist protein and soluble TNF receptor

(reviewed by D. H. Lewis, Chan, Pinheiro, Armitage-Chan, & Garden, 2012). The induction of suppression-related responses may represent a proactive attempt by the host to moderate excessive inflammation. A functional impact of early IL-10 in UPEC UTI mice model were suggested as IL10$^{-/-}$ mice had more UPEC and more severe bacteriuria (Duell et al., 2012). Interestingly, Pyo18 down-regulated *IL-10* transcription, whereas Pyo18*ΔhlyA* up-regulated gene transcription. The observed down-regulation in *IL-10*, *TNFα* and *IL-1β* transcription in stromal cells may be seen as an attempt of hemolytic *E. coli* to delay the activation of the immune response.

*TRAM*, *TRIF* and *IRF3* transcripts were constitutively detected in healthy diestrous and pyometra endometria. Transcription of *INFβ* was not detected in endometria (Experiment 1), but was detected in endometrial epithelial cells (Experiment 2) after incubation with Pyo18, Pyo18*ΔhlyA*, Pyo14 and LPS. The TICAM/TRIF pathway considers the phosphorylation of IRF3, which enters the nucleus and triggers the transcription of interferon encoding genes such as *IFNβ* and *IFNγ* (reviewed by Lewis et al., 2012) and the late-phase activation of NFkB and MAPK. The increase transcription of *IFNβ* by endometrial epithelial cells after 1h of bacterial stimuli is likely triggered by LPS and is probably responsible for the up-regulation of *CXCL10* transcription after 4h of stimulation. The lack of transcription of *IFNγ* in normal diestrus and pyometra uteri is in accordance with the results of endometrial transcription and serum levels of IFNγ found in pyometra cases (Hagman et al., 2009; Karlsson et al., 2012).

In conclusion, β-hemolytic *E. coli* infection was associated with the occurrence of metritis and with an higher uterine tissue damage than infection by non-hemolytic *E. coli* strains. β-hemolytic *E. coli* pyometra endometria had higher gene transcription of *IL-1β* and *IL-8* and lower gene transcription of *IL-6* than non-hemolytic pyometra endometria. The cytotoxic effect of α-hemolysin was more severe in endometrial stromal than in epithelial cells. Endometrial stromal cell damage by α-hemolysin is a potential relevant step of *E. coli* virulence in the pathogenesis of pyometra. Additionally, inhibition of cytokine production by α-hemolysin, which may occur during the early stages of infection, may allow bacteria to establish a niche prior to the activation of an adequate innate immune response. Low levels of pro-inflammatory cytokines IL-1β and TNFα may compromise the chemotaxis and activation of immune cells, leading to a precocious high level of cell and tissue damage. Down-

regulation of IL-10 production might be associated with an excessive inflammatory reaction, thus enhancing tissue damage.

# CHAPTER VII - GENERAL DISCUSSION, CONCLUSONS AND FUTURE PERSPECTIVES

## General Discussion

Pyometra is a common and important uterine disorder that affects adult bitches, causing a variety of clinical and pathological signs. In fact, around 25% of all intact diestrous female dogs before ten years of age develop pyometra (Egenvall et al., 2001). An overall annual pyometra incidence of 2% was determined (Egenvall et al., 2001). Despite several studies on the etiology of the disease, the specific pathogenesis of pyometra is still not completely understood. Pyometra develops as a result of a complex interaction of etiological physiopathological factors. These factors include changes within the endometrium, the hormonal influence on uterine environment, the virulence and type of the bacteria, and the individual defense mechanisms (Mateus & Eilts, 2010).

Bacterial uterine infection in dogs has many similarities with severe bacterial infections in humans. For example, infection in both species is associated with induction of local and systemic inflammation, cytokine production, an acute phase reaction, endotoxemia and induction of subsequent sepsis. *Escherichia coli* is the most common bacterium isolated from the uterus of bitches with pyometra (Chen et al., 2003; Mateus et al., 2013; Wadås et al., 1996) and its presence is normally associated with highly severe systemic signs. If left untreated is lethal and patients may develop endotoxemia, sepsis or septic shock (Fransson et al., 2007). In fact, 50% of canine *E. coli* pyometra cases are associated with SIRS (Fransson et al., 2007). Therefore, an examination of disease mechanisms involved in pyometra may also provide important insights to the mechanisms operating during human bacterial infection and sepsis. Also, an enhanced understanding of innate immune mechanisms within the female reproductive tract and their role in bacterial recognition may provide insight into the pathogenesis of uterine diseases and the deleterious sequelae associated with genital tract inflammation (Chapters III and VI).

In dogs, uropathogenic *E. coli* (UPEC) strains are the most frequently isolated pathogens in canine urinary tract infections - UTI (53.9-68%) with approximately 14% of all dogs having at least one episode of UTI during their lifetime (Windahl, Holst, Nyman, Grönlund, & Bengtsson, 2014). However, UPEC strains are implicated in up to 90% of pyometra cases (Chen et al., 2003; Mateus et al., 2013; Wadås et al., 1996). One possible hypothesis is that pyometra and UTIs *E. coli* share VF, that enable them to colonize and

infect either the uterus or the urinary tract. In order to answer respond this question, canine *E. coli* strains from pyometra, cystitis and fecal origin were compared in terms of prevalence of different VF genes and UPEC PAIs (Chapter IV).

Canine UPEC *E. coli* were found to be similar to human uropathogenic strains with regard to their serotypes, clonal types, phylogenetic background and VF profile (Beutin, 1999; Johnson et al., 2008). This leads to the suggestion that dogs may act as reservoirs of potential virulent bacteria that can be transmitted to humans (Johnson et al., 2001a). This is important because of the potential implications to prevent against extraintestinal (ExPEC) *E. coli*, and because of the close contact between humans and their pets. In fact, within-household sharing of clonal fecal *E. coli* was verified in humans and their pets in two longitudinal case studies (Johnson & Clabots, 2006; Sannes, Kuskowski & Johnson, 2004). An enhanced knowledge of the importance and the role of specific *E. coli* VF may lead to further research into the design of novel approaches to the prevention of ExPEC diseases as UTI in dogs and humans and pyometra in dogs.

*E. coli* is also the most prevalent bacterium isolated during the first week postpartum from the uterus of cows that developed puerperal uterine infection (Bicalho et al., 2012; Mateus et al., 2002; Sheldon et al., 2009) and around 40% of lactating cows may develop metritis within two weeks of calving (Sheldon, Williams, Miller, Nash, & Herath, 2008). However, the association between *E. coli* virulence traits and the development of puerperal or clinical metritis is controversial (Bicalho et al., 2012; Bicalho et al., 2010; Sheldon et al., 2010; Silva et al., 2009a). In the puerperal cow, *E. coli* favors the establishment of *Trueperella pyogenes* and Gram-negative anaerobes in the uterus, inducing an infection that is usually associated with abnormal postpartum ovarian function and impaired reproductive performance (Mateus et al., 2002; Sheldon et al., 2009). However, in the cow, uterine infection may resolve spontaneously (Mateus et al., 2002). In an attempt to correlate the virulence potential of uteropathogenic *E. coli* with the different clinical outcomes of canine pyometra and bovine postpartum metritis we further characterized uteropathogenic *E. coli* VF traits (Chapter V). Until the present, studies on canine pyometra did not take into account the virulence background of *E. coli*. The observed high prevalence of β-hemolytic *E. coli* in cases of pyometra (Chapter IV) leads us to the suggestion that α-hemolysin contributes to the virulence of *E. coli* strains. Therefore, in Chapter VI, we evaluated the role of *E. coli* α-hemolysin in the pathogenesis

of canine pyometra, and on the immune response of canine endometrial epithelial and stromal cells.

The extraintestinal pathogenic *E. coli* strains usually belong to groups B2 and D (Picard et al., 1999), the commensal strains to groups A and B1 (Bingen et al., 1998), whilst the intestinal pathogenic strains belong to groups A, B1 and D (Pupo et al., 1997). In general, strains from phylogenetic groups B2 and D contained more virulence factors than strains from the groups A and B1 (Johnson & Stell, 2000b). In fact, in our work, independently of their origin, *E. coli* isolates from phylogenetic group B2 had higher number of VF genes then the isolates from groups A, B1 and D (Chapter IV), and consequently have a higher potential of virulence. The prevalence of group B2 was higher in *E. coli* isolates from pyometra (94%) than from cystitis (48%) or fecal origin (39%) (Chapter IV), indicating that group B2 has the highest potential to colonize the canine uterus and establish infection. An important finding is that *E. coli* from B2 group were highly represented in canine faeces, which may provide a reservoir of ExPEC for humans or receptive dogs (Johnson et al., 2001a). Interestingly, uteropathogenic bovine *E. coli* strains were mainly assigned to groups B1 and A, and showed a distinct VF-gene profile, evidenced by the cluster analysis based on virulence traits (Chapter V). Bovine and canine *E. coli* isolates presented a low degree of genetic similarity (28%), indicating host species-specific genomic profiles. Also, the high genomic diversity observed among *E. coli* isolates of both host species suggests that in each disease, the isolates do not originate from a specific clone that is epidemically spread between animals, as also suggested by Hagman & Kühn (2002) and Silva et al. (2009a). The low virulence potential here observed for *E. coli* strains isolated from bovine metritis cases is in accordance to the so far known role of this bacterium in the pathogenesis of metritis and may be related with the short persistence of the bacterium in the uterus during the puerperal period, even in the case of metritis (Mateus et al., 2002; Sheldon et al., 2009). Although metritis *E. coli* isolates lack the common pathogenicity genes associated with virulence of ExPEC, we cannot forget that in the postpartum uterus the epithelial barrier is already broken, given open access to bacteria to other endometrial cell compartments. Also, puerperal cows are characterized by an immunosuppressive state, which predispose to uterine infection. In the bitch, the high endogenous progesterone concentration in the first half of diestrus impairs antimicrobial defenses (Faldyna, Laznicka, & Toman, 2001; Kida et al., 2006; Silva et al.,

2012) and predisposes to endometrial *E. coli* adherence (Ishiguro et al., 2007). Besides the above factors, the exuberant clinical signs associated with pyometra may be related with the high virulence profile of *E. coli* strains isolated from affected bitches. This virulence potential may enable *E. coli* to be the solo bacterium in the pathogenesis of canine pyometra. Also LPS has an important role in both diseases. In bitches with pyometra, a high blood concentration of endotoxins was related to poor prognosis (Karlsson et al., 2012). In the cow, concentrations of endotoxin and PGE2 in the uterine fluid were related to the severity of bovine metritis, and absorption of endotoxin from the uterus was associated with ovarian dysfunction (Mateus et al., 2003).

Genome size differs among the phylogenetic groups, with A and B1 strains having smaller genomes than B2 or D strains (Bergthorsson & Ochman, 1998). This fact may explain, in part, the observed lower antibioresistance of B2 strains (Chapter V). The prevalence of resistance towards two or more antimicrobials was lower in *E. coli* strains belonging to phylogenetic group B2 than in strains belonging to phylogenetic groups B1 and A, reinforcing the idea of a trade-off between resistance and virulence (Cooke et al., 2010; Houdouin et al., 2006; Johnson et al., 2005c). Overall, resistance to antimicrobials currently used in the treatment of pyometra and metritis was low. The most frequent antimicrobial resistance of canine pyometra *E. coli* strains were towards amoxacillin/clavulanic acid (14%) and ampicillin (17%), the most commonly used antimicrobial drugs for the treatment of uncomplicated cases of canine pyometra. A similar prevalence of resistance to the above antimicrobials was reported by Hagman & Greko (2005). Only 3% of the canine *E. coli* isolates were not susceptible to enrofloxacin, an antimicrobial currently used for treatment of complicated pyometra cases and UTIs. Since UTI and pyometra may coexist in the same animal, enrofloxacin may be the antimicrobial of choice before antimicrobial susceptibility is confirmed. In the puerperal cow, tetracycline is widely used for both therapeutic and prophylactic purposes. This is probably the main reason for the occurrence of the low susceptibility observed by us (35%) and others (Sheldon et al., 2004b). The high susceptibility of bovine *E. coli* strains to ceftiofur reflects the good efficacy of this antimicrobial in the treatment of metritis and clinical endometritis (Galvão et al., 2009; Sheldon et al., 2004b). Companion and food animals may act as reservoirs of antimicrobial-resistant ExPEC that can be transmitted to humans (or vice versa) by direct contact (petting, licking, physical injuries) or indirectly,

via the food chain (Guardabassi et al., 2004; Johnson et al., 2001a; Marshall & Levy, 2011; Stenske et al., 2009). Therefore, the evaluation of antimicrobial resistance patterns is useful in the establishment of appropriate therapeutic measures that avoid the emergence of resistant *E. coli* strains potentially harmful to humans and other animals.

Virulence factors of *E. coli* that have been potentially implicated as important to the establishment of uterine or urinary tract infection can be divided into two groups: (i) virulence factors associated with the surface of bacterial cell and (ii) virulence factors, which are secreted and exported to the site of action (Emody, Kerényi, & Nagy, 2003). The bacteria adhesion to host cells is a crucial step of the infection. *fimH* gene, encoding type 1 fimbriae, was the most prevalent adhesin gene in pyometra *E. coli* isolates, being detected in all isolates. However, the role of the type 1 fimbriae in pyometra is difficult to define because 95.7% and 88.5% of *E. coli* from UTI and from fecal origin, respectively, also harbored this gene. The functional significance of *fimH* gene presence is also controversial, as *fimH* expression is regulated by environmental conditions and mannose receptors availability (Schwan, 2011). In addition to *fimH*, pyometra *E. coli* isolates carried several other adhesin genes. However, none of the screened genes were specifically associated with these isolates, when compared with cystitis and fecal isolates. The genes coding for P and for F1C fimbriae were detected in a high prevalence in *E. coli* pyometra isolates (48.4% for *papEF* and 61.3% *focG*) suggesting that these genes may also have a role in the bacterial adherence to endometrial cells. By contrast, all bovine *E. coli* isolates harbored *fimH* gene, *P* and *F1C* coding adhesin genes were not detected in the genome of these isolates. The high prevalence of several adhesin genes in pyometra *E. coli* isolates may indicate functional redundancy among adhesins, as previously suggested (Krekeler et al., 2013). In fact, although adherence to bovine (Sheldon et al., 2010) and canine (Krekeler et al., 2013) endometrial cells was shown to be mediated by Type 1 fimbriae, targeted deletion of specific adhesin genes in a canine pyometra strain was compensated by the presence of other adhesins (Krekeler et al., 2013). In accordance, in our study, no differences in adhesion levels were obtained when endometrial cells were incubated with *E. coli* with different adhesins profiles.

The *hlyA* and *cnf1* genes, coding for α-hemolysin and cytotoxic necrotising factor-1, respectively, were also highly prevalent (48.8%) in pyometra isolates, which is in agreement with previously reported results (Chen et al., 2003; Siqueira et al., 2009). These

toxins may have an important role in the progression of the disease as they are classically associated with an increase in tissue damage, inflammation and destruction of immune cells, which favors bacterial survival and invasion (reviewed by Bien et al., 2012). However, the precise role of HlyA and CNF1 in the pathogenesis of canine pyometra is still unknown.

The detection of multiple iron acquisition system genes (*fyuA, iucD, iroN, fepA, sitA* and *chuA*) in canine and bovine isolates suggests a functional redundancy, but also an advantage for bacteria colonization and adaptation to different environments. The higher prevalence of *fyuA* and *chuA* genes in pyometra isolates leads to the suggestion that these systems have a greater impact on uterine colonization, as observed for siderophore receptors yersiniabactin (FyuA) and aerobactin (IutA) in the urinary tract (reviewed by Garénaux et al., 2011). Besides its role in iron uptake, yersiniabactin also interferes with the host response and innate immune system, being characteristic of bacteriemic *E. coli* (Johnson & Stell, 2000b).

Many VF genes of pathogenic *E. coli* strains are clustered in the chromosome in "pathogenicity islands" (PAIs). Although pyometra, cystitis and fecal isolates can have similar virulence profiles, the prevalence of classical UPEC PAI markers was significantly higher in pyometra isolates (100%), than in cystitis (74%) and fecal (54%) isolates. The high prevalence of PAI markers detected in pyometra isolates is also associated with a high prevalence of the phylogenetic group B2 background among these isolates. We also found a high number of pyometra isolates harboring multiple PAIs which indicate that these isolates possess several combinations of VFs conferring them a selective advantage during adaption and colonization of a diverse set of environments. Moreover, as the same VF can be encoded by different PAIs, the isolates with multiple PAIs can lose one PAI and acquire new VFs with no effect on phenotype. In our study, this was evident for a-hemolysin, which is encoded by three PAIs (I536, II536 and IIJ96). The relative prevalence of some PAIs may also reflect their genomic stability. In accordance, PAI IV536 and PAI III536 were respectively, the most and the least prevalent PAIs in the three groups of isolates. However, PAI IV536 and PAI ICFT073 were significantly more prevalent in pyometra than in cystitis and fecal isolates, indicating that these PAIs are potentially relevant for *E. coli* virulence in pyometra. Our screening of potential VF genes and PAIs in the three groups of isolates revealed that some of the genes were physically

linked within the PAIs. This was the case of the PAI IIJ96 that classically carries the genes for α-HlyA, Prs fimbriae and CNF1. Our results showed that in 96% of the cases the presence of PAI IIJ96 was always associated to the presence of *cnf1* and *hlyA* genes and to *papEF* gene. Overall, our study shows that pyometra *E. coli* isolates have a high virulence potential that can also be found in a subset of fecal and cystitis isolates. This leads to the suggestion that these isolates may be able to induce pyometra in receptive hosts. In accordance, in cases of *E. coli* pyometra with a concurrent subclinical urinary tract infection, the urinary tract and the uterus are likely to be infected by the same bacterial strain (Hagman & Kühn, 2002; Wadås et al., 1996).

Results presented in Chapter III showed that TLR-mediated immune surveillance is an important component of the defense mechanisms within the canine uterus. Transcription of TLR1-7 and TLR9 genes was detected in the canine endometrium in all phases of the oestrous cycle. This observation indicates that canine endometrium is able to recognize a large variety of pathogen-associated molecular patterns (PAMPs) and to orchestrate an innate immune response against bacterial and viral pathogens. TLR1–10 genes were also detected in the uterus during the human menstrual cycle (Aflatoonian et al., 2007; Schaefer et al., 2004), and in bovine (Davies et al., 2008) and mouse endometrium during the oestrous cycle (Yao, Fernandez, Kelly, Kaushic, & Rosenthal, 2007). Activation of these genes constitutes a well-conserved pathway for triggering the inflammatory response within the uterus. Of the 13 described mammalian TLRs (Yamamoto & Akira, 2005), TLR2 and TLR4 are the best characterized with respect to innate immune responses to bacteria. Differential endometrial transcription and expression of TLR2 and TLR4 occurred during the oestrous cycle, indicating a regulatory role of ovarian steroids (Chapter III). The high expression of TLR2 and TLR4 observed during the follicular phase might be related with the uterine contamination normally occurring during proestrus and oestrus, resulting from ascending bacteria from the vagina (Kustritz, 2006).

*Escherichia coli* pyometra develops in the first half of dioestrus under high endogenous progesterone concentrations (Tsumagari et al., 2005). This fact seems to be related to an impairment of the antimicrobial defenses (Kida et al., 2006) and to an increase in endometrial *E. coli* adherence (Ishiguro et al., 2007). The fact that TLR2 and TLR4 transcription and expression are the lowest during early diestrus in association with the low numbers of endometrial leucocytes observed in non-pathological diestrous uteri

(Chapter VI) probably reflects the suppression of the immune system by progesterone (Faldyna et al., 2001; Kida et al., 2006). This could potentially be associated with the increased susceptibility to pyometra observed at this stage. However it might be favourable to implantation by reducing the inflammatory reaction against the conceptus. An important aspect is that TLR2 and TLR4 signaling components are constitutively transcribed in diestrous uteri, which prompt the uterus to sense and rapidly mount an immune response following a pathogen insult (Chapter VI).

Dissociation between protein expression and gene transcription of both TLRs was evident during late dioestrus and anoestrus, which may be associated with post-transcriptional regulation. The higher transcription levels of TLR2 and TLR4 during these phases, compared with the follicular phase, might be linked to the high number of macrophages present in the endometrium at those stages, due to the high apoptotic index of the stromal and endothelial cells (Henriques et al., 2015). One of the cellular downstream products of TLR signaling is prostaglandins (PG) synthesis (Helliwell et al., 2004). In pyometra endometria, prostaglandin synthesis genes are upregulated and endometrial concentrations of PGE2 and PGF2, were high, which could further regulate the local inflammatory response (Silva et al., 2009b; Silva et al., 2010). The up-regulation of these genes in pyometra cases probably also reflect the high infiltration of leukocytes, mainly neutrophils and macrophages, reported in Chapter VI. The higher PGE2 content measured in pyometra endometria most likely results from the synchronized up-regulation of PTGS2 and PGES after endotoxin stimulation, as demonstrated by others (Helliwell et al., 2004). The high uterine concentrations of PGE2 could further contribute to the suppressed activity of cellular immunity during diestrus. PGE2 is known for its immunosuppressive effect and the immunomodulatory role of PGE2 is observed on lymphocytes, monocytes/macrophages and PMN (Rocca & FitzGerald, 2002). The high PGF2α endometria content can justify the higher systemic concentrations of PGFM observed in bitches with pyometra (Hagman et al., 2006b). Also it can be responsible for the early luteolysis observed in *E. coli* pyometra cases. In the bitch, around 60% of pyometra cases are associated with serum P4 concentrations lower than 1 ng/L at the time of diagnosis (England et al., 2007; Fieni, 2006), whereas in the normal mid-dioestrus P4 concentrations are around 10–30 ng/L. In order to simulate in vitro the in vivo endometrial exposure to Gram-positive and -negative bacteria, endometrial explants were stimulated with LTA and

LPS, and PGF2α and PGE2 were measured in the culture medium. Regardless of the oestrous cycle phase, endometrial explants produced higher concentrations of PGF2α than of PGE2, as also reported for pyometra cases (Silva et al., 2010). Interestingly, this high ratio of PGF2α:PGE2 was not observed in bovine and ovine endometrial explants after stimulation with LPS (Herath et al., 2009a; Vagnoni et al., 2001). In the cow, an increase in the uterine fluid PGE2 concentrations during the puerperium was associated with the persistence of the uterine infection (Mateus et al., 2003) and the prolonged lifespan of the corpus luteum (Herath et al., 2009a).

Results from chapter VI showed that β-hemolytic *E. coli* pyometra was characterized by high endometrial damage and metritis. Also, higher endometria transcription levels of IL-1β and IL-8 and lower transcription levels of IL-6 were observed in hemolytic *E. coli* pyometra than in non-hemolytic *E. coli* pyometra. However, clinical signs, hematological and blood biochemical results were similar in both groups. These results may reflect the late diagnosis of the disease and the effect of *E. coli* LPS in different organs, rather than the direct effect of α-hemolysin. One of the reasons for the severe endometrial damaged observed in pyometra cases is the high inflammatory cell infiltration. IL-6 stimulates leukocyte activation and myeloid progenitor cell proliferation, regulates the synthesis of acute phase proteins and is a powerful pyrogen (Scheller et al., 2011). In pyometra cases, C-reactive protein, serum amyloid A and haptoglobin concentrations can be used to monitor early post-OVX complications and ongoing inflammation (Dabrowski et al., 2009). C-reactive protein was also associated with SIRS (Fransson et al., 2007). High serum levels of IL-8 were observed in pyometra bitches, especially in those that developed SIRS, suggesting IL-8 as a useful early biomarker of uterine infection and, possibly, of sepsis (Karlsson et al., 2012). IL-8 is also a potent neutrophil chemotactic molecule. Neutrophil recruitment to the site of infection was shown to be critical for bacterial clearance in the uterus. However, their action may also lead to tissue damage (Kruger et al., 2015). Our results showed that *E. coli* α-hemolysin also contributes to tissue damage due to their cytotoxic activity, mainly directed towards stromal cells. This facilitates progression of bacteria into the uterine tissue. The sensitivity of both epithelial and stromal cells to the cytotoxic effect of HlyA may explain the observed significant association between β-hemolytic *E. coli* isolation and metritis. Additionally, the known cytotoxic effect of hemolysin in granulocyte and lymphocytes as well as the enhancement

of access to cell nutrients and iron storages (reviewed by Bien et al., 2012) might also enhance hemolytic *E. coli* survival within the uterine tissue.

Results presented in chapter VI also indicate that α-hemolysin contributes to the virulence of *E. coli* through the modulation of the uterine innate immune response. In stromal cells, Pyo18 decreased transcription level of IL-1β, TNFα and IL-10 compared to the isogenic mutant Pyo18*ΔhlyA* and to Pyo14. This may be seen as an attempt of hemolytic *E. coli* to delay the activation of the immune response. As a result, a delayed neutrophil influx to the uterus may allow *E. coli* to establish in the uterus leading to a precocious higher level of cell and tissue damage. The induction of suppression-related responses may represent a proactive attempt by the host to moderate excessive inflammation. Hence, delay in IL-10 production might be associated with an excessive inflammatory reaction, thus enhancing tissue damage.

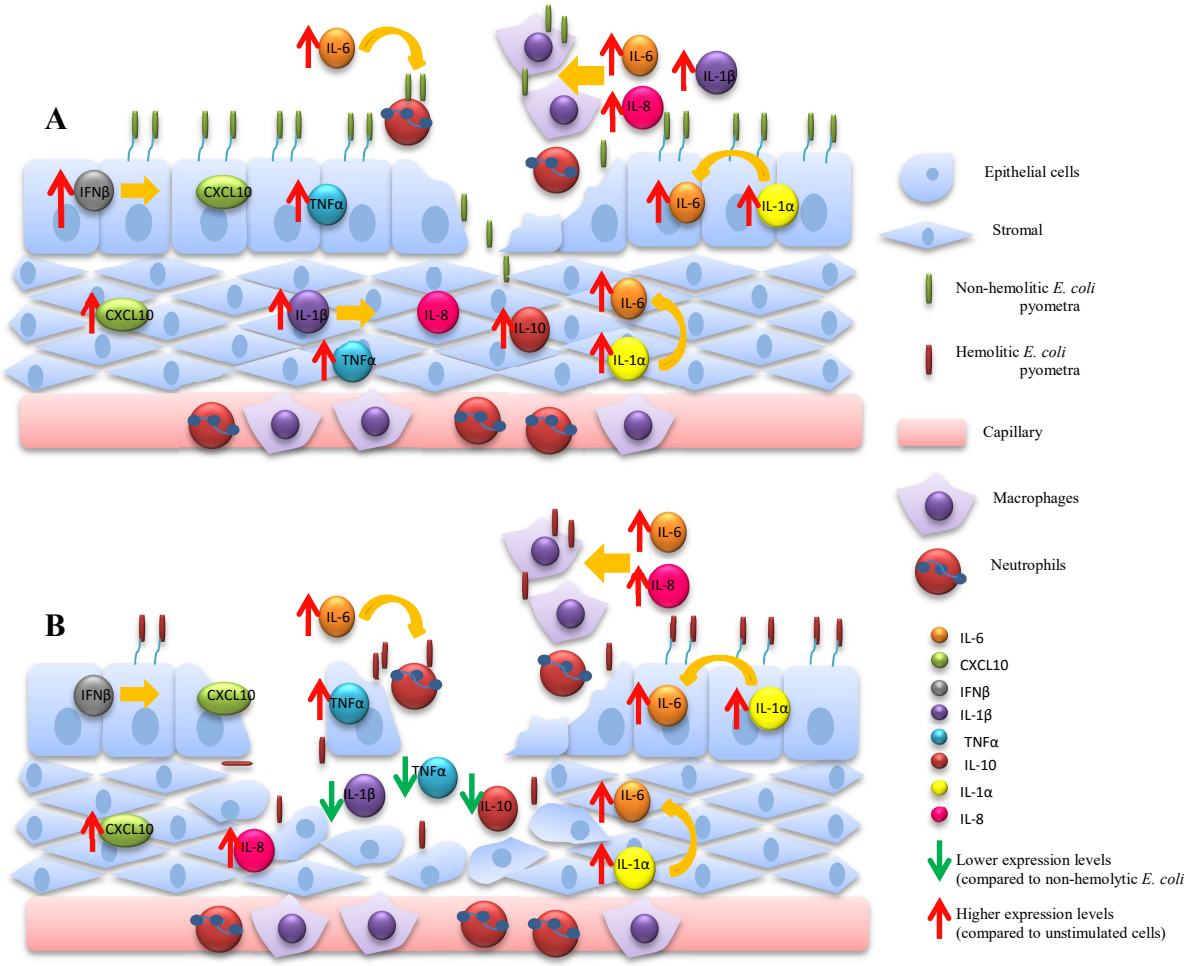
Endometrial epithelial adherence is critical for establishment of uterine infection. We showed that uteropathogenic *E. coli* strains possess different types of adhesins (Chapter IV and V). Consequently, the first line of host defense against uterine infection is concentrated on preventing bacteria adherence to the uterine mucosa. Two of the components of this defense line in canine uterus are mucin I (Ishiguro et al., 2007) and defensins (Krekeler, 2011). CXCL10 has antimicrobial properties similar to those of defensins (Gallo et al., 2002) and was demonstrated to have antimicrobial activity against *E. coli* (Cole et al., 2001). In our study, a strong up-regulation of CXCL10 was observed in canine endometrial epithelial cells after 4h incubation with *E. coli* (Chapter VI). As reported by Karlsson et al. (2015), CXCL10 was also up-regulated in endometrial stromal cells. Epithelial and stromal cells may participate in the recruitment of T cells, during bacterial infection of the uterus, since this chemokine is also involved in the recruitment and potentiation of T-helper 1 response (Dufour et al., 2002). In contrast to CXCL10, a higher transcription level of IL-8 was observed in stromal compared to epithelial cells. Stromal cells were also responsible for a high level of IL-1β mRNA expression. Our data leads to the suggestion that stromal cells may be an important early source of IL-1β and IL-8 for neutrophil and macrophages recruitment during bacterial infection of the uterus, after disruption of the epithelial layer of the endometrium.

The role of MyD88 independent pathway activation in pyometra is less understood. Although, TRAM, TRIF and IRF3 transcripts were constitutively detected in pyometra

and normal uteri, INFβ and IFNγ transcription was not detected, which is in accordance with results by Hagman et al. (2009) in pyometra uteri or in the blood of bitches with pyometra (Karlsson et al., 2012). However, in our study, an increase in INFβ gene transcription was observed in epithelial cells after 1h incubation with both bacteria and LPS, which is in agreement with IRF3 activation in our culture system, and may be responsible for the up-regultion of CXCL10 mRNA at 4h in the same type of cells (Chapter VI).

The proposed role of α-hemolysin is schematically summarized in figure 20.

**Figure 20 - Schematic illustration of the proposed initial endometrial inflammatory events following infection with non-hemolytic (A) and hemolytic *Escherichia coli* (B) strains**



Non-hemolytic *Escherichia coli* (*E. coli*) do not induce significant epithelial and stromal damage. Access to the stroma occurs in a dispersed manner along the epithelium (A). In contrast, hemolytic *E. coli* induce extensive epithelial and stromal damage leading to invasion of deeper compartments and causing metritis (B).
In endometrial epithelial and stromal cells, both *E. coli* strains are sensed by TLRs which activates the NFκB and IRF3 pathways. This leads to overexpression of a panel of interleukins, chemokines and interferon type I genes that are involved in the inflammatory response (see chapter X) (A, B). However, in stromal cells, hemolytic *E. coli* induces a lower *IL-1β*, *IL-10*, *TNFα* gene transcription (B), which might delay the activation of the immune response.

## Conclusions

The results presented in this thesis contributed to a more comprehensive understanding of the pathogenesis of canine pyometra. Overall, these findings provide new relevant insights into the role of the pathogen-specific modulation of host immunity, which may influence the severity of disease and its clinical outcomes
More specifically:

1. TLRs are involved in the activation of the inflammatory response associated with pyometra in the bitch. TLRs may therefore be therapeutic targets for the control of uterine bacterial infections in the bitch and potentially in other species.
2. *Escherichia coli* strains isolated from pyometra cases have a high potential of virulence and a broad virulence genotype, although being similar to a subset of cystitis and fecal isolates. This leads to the suggestion that cystitis and fecal isolates may be able to induce pyometra in receptive hosts.
3. β-hemolytic *E. coli* infection was associated with the occurrence of metritis and with an higher uterine tissue damage.
4. α-hemolysin contributes to the virulence of β-hemolytic *E. coli*, by inducing endometrial epithelial and stromal damage and a compromised early uterine immune response.

## Future Perspectives

Future studies should further assess how different *E. coli* strains influence disease progression and outcome. Also, the characterization of the role of other specific *E. coli* VF genes in the modulation of the endometrial immune response and on the pathogenicity of the bacterium may prove rewarding in the development of novel diagnostic and therapeutic approaches to the disease. For example, the development of vaccines which target the mechanisms by which *E. coli* adhere to the mucosal epithelium, such as fimbrial adhesins, is being considered a promising strategy for preventing UTIs (Langermann et al., 1997; Langermann & Ballou, 2001; Schembri, Kjaergaard, Sokurenko & Klemm, 2001). Likewise, development of

vaccines targeted to induce an immunity that can block the binding and action of microbial toxins, such as HlyA (Holmgren, Czerkinsky, Eriksson, & Mharandi, 2003) can be rewarding. Additionally, treatment with selective ciclooxygenase-2 inhibitors could be an interesting joint-option, to control the inflammatory response.

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