

# *Escherichia coli* virulence influences the roles of sex hormone receptors in female dogs with simulated pyometra

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**Abstract.** The present study aimed to investigate whether *Escherichia coli* virulence affects the roles of sex hormone receptors in female dogs with simulated pyometra. A total of 33 healthy, nulliparous, crossbred female dogs were divided into four groups, with 10 dogs in each of the three experimental groups and 3 dogs in the control group. Estradiol was administered to female dogs in group 1 continuously at 0.6-4.8 mg/kg twice daily for 12 days (the dose doubled every three days), followed by intramuscular injection of 0.2-1.8 mg/kg progesterone. The progesterone was administered with an initial dose of 0.2 µg/kg and increased 0.2 mg/kg every three days, twice daily until the maximum of 1.8 mg/kg for 24 days and maintained at 1.8 mg/kg for 19 days. Progesterone only was administered at 1.8 mg/kg in group 2 (twice daily) for 55 continuous days and only estradiol was administered with an initial dose of 0.6 µg/kg (dose doubled every 3 days for 12 days) in group 3 twice daily and maintained at 4.8 mg/kg for the following 43 days. A strongly virulent *E. coli* strain, nau-b, and a weakly virulent strain, nau-i, were screened. On the 12th day of diestrus, 5 female dogs in each of the experimental groups were inoculated with *E. coli* nau-i strain, while the other five in each group were inoculated with nau-b strain. Histopathological changes of uterine tissues were microscopically observed 50 days after *E. coli* inoculation and hormone receptor expression levels were detected by quantitative polymerase chain reaction. Simulated pyometra was observed in dogs administered with progesterone alone or progesterone combined with estradiol. The clinical symptoms and histopathological observation demonstrated that inoculation with strongly virulent *E. coli* strain, nau-b, caused earlier onset of pyometra symptoms and more severe pyometra

symptoms compared with the weakly virulent *E. coli* strain, nau-i. Furthermore, estrogen and progesterone receptor levels in dogs with pyometra inoculated with *E. coli* strain nau-i was higher than those in dogs with pyometra inoculated with *E. coli* strain nau-b. These results indicated that *E. coli* affects the roles of sex hormone receptors in female dogs with simulated pyometra.

## Introduction

Pyometra is a common, metoestral disease in adult, ovary-intact female dogs, which is characterized by endometrial hyperplasia and infiltration of inflammatory cells (1,2), with typical manifestations of lethargy, depression, loss of appetite, vomiting, thirst, polydipsia, polyuria and progressive (tremor) shock (3). The detailed mechanisms underlying its pathogenesis are not fully understood; however, it is believed to be principally attributed to primary hormonal imbalance and secondary bacterial infection (4).

Pyometra predominantly occurs during the metoestrus phase of the estrous cycle, when progesterone is the dominant hormone (5,6). Progesterone may induce endometrial proliferation and uterine glandular secretion, decrease myometrial contraction and induce closure of the cervix (7). Progesterone also inhibits the bactericidal activity of the uterus against *Escherichia coli*, while estrogen stimulates the clearance of bacteria (8). Estrogen increases the uterine sensitivity and reaction to progesterone (7). The circulating exogenous or endogenous estrogen concentration affects the distribution of estrogen receptors (ERs) within the uterus of female dogs. Notably, ERs and progesterone receptors (PRs) have also been implicated in the pathogenesis of pyometra, as alteration in their expression has been observed in the uterine wall of female dogs with pyometra (9,10), although the changes are not consistent among different studies (9,10).

*E. coli* is the bacterial agent most commonly isolated from the infected uterus in female dogs with pyometra (11), and the strains are clonally related to those isolated from urinary tract infections (12). Furthermore, it has been reported that the epithelium and endometrium of the urinary tract have affinity to *E. coli* when sensitized by a high level of progesterone (13). Previous studies of *E. coli* isolated from canine pyometra have indicated a variable frequency of virulence genes encoding adhesins (including *fimH*, *papC* and *papGIII*),

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toxins (including *hlyA/F*, *usp* and *astA*) and other factors (such as those involved in escape from host defenses and iron acquisition) (11,14,15).

The aim of the present study was to investigate whether *E. coli* virulence affects the roles of sex hormone receptors in female dogs with simulated pyometra. The study was designed to provide more information about the roles of *E. coli* and hormone receptors in the etiology of pyometra.

## Materials and methods

**Isolation and identification of *E. coli* strains.** About 1-3 ml pus was collected from each uterus (total 26 uteri) that were came from female dogs undergoing surgery to remove the uterus, diagnosed with canine pyometra at Nanjing Agricultural University Animal Hospital (Nanjing, China). The pus sample was first incubated in Lysogeny broth (LB; BioSyntech Inc., Beijing, China) at 37°C for 24 h, followed by subsequent incubation in MacConkey medium (BioSyntech Inc.) at 37°C for 24 h. A single red smooth colony was stained with Gram's stain and then examined under a light microscope (Olympus CX23; Olympus Corporation, Tokyo, Japan). The red, short, bacilliform bacteria were selected and first inoculated into LB (1:100 dilution) at 37°C for 24 h and then subcultured at a dilution of 1:100 in eosin-methylene blue medium at 37°C for 24 h. A single black colony with metallic luster was stained with Gram's stain and then examined under a light microscope. The red, short, bacilliform bacteria were considered to be *E. coli* isolates, which were then inoculated into LB (1:100 dilution) at 37°C for 24 h, and subsequently on MacConkey medium (1:100 dilution) at 37°C for 24 h.

Biochemical identification of *E. coli* was performed using the national standard methods, as previously described (16). Only isolates meeting the following criteria were considered to be *E. coli*: i) Positive results in the indole and methyl red tests; ii) negative results in the Voges-Proskauer and citrate tests; and iii) producing acids or alkali, with or without H<sub>2</sub>S on the slants, while producing acids on the slants without H<sub>2</sub>S at the bottom in the triple sugar iron agar slant test. Finally, the cultures were stored at -70°C after mixing with 50% glycerol at 1:1.

**Detection of virulence genes in *E. coli* strains.** *E. coli* DNA was isolated using the OMEGA Bacterial Genomic DNA Extraction kit (Omega Bio-Tek, Inc., Norcross, GA, USA), according to the manufacturer's instructions. The extracted DNA was then stored at -20°C for later use. Polymerase chain reaction (PCR) performed on ABI Prism 7300 Detection System (Applied Biosystems Inc., Carlsbad, CA, USA) was used to detect seven major genes causing urinary tract infection in the isolated *E. coli* strains: *hlyA*, *sfa*, *fim*, *pap*, *papC*, *papGIII* and *usp*. Specific primers (Table I) were designed with reference to the conserved sequences described in previous studies (15,17). GAPDH was used as a control gene, and the sequence of GAPDH primer was as follows: Forward 5'-ATG ACTATCAAAGTAGGTATCA-3', reverse 5'-TTATTTGGA GATGTGAGCGATCAG-3'. PCR reactions were performed in a 25 µl reaction volume containing 12.5 µl PCR MasterMix (Biotek Corporation, Beijing, China), 1 µl upstream and downstream primer each, 8.5 µl ddH<sub>2</sub>O and 2 µl DNA

template. PCR products were identified by 1.0% agarose gel electrophoresis. The amplification conditions was as follows: 30 cycles of 94°C for 1 min, annealing at specific temperatures for each gene (Table I), 72°C for 2 mins and 72°C for 7 mins. The experiment was repeated three times.

**Determination of the half lethal dose (LD<sub>50</sub>) of the isolated *E. coli* strains.** A total of 250, 4-week old ICR mice (18-22 g) of 125 male and 125 female, 6-8 weeks old, were obtained from Laboratory Animal Center & Institute of Comparative Medicine of Yangzhou University (Yangzhou, China). The animals were maintained on a 12-h-dark/12-h-light cycle at ~20°C and 50-60% humidity, with *ad libitum* access to food and water during the experiments. The ICR mice were randomly divided into 10 groups of 25 animals each, including 9 experimental groups and one control group. The 9 experimental groups were each administered a different strain of *E. coli*; nau-h, nau-d, nau-o, nau-q, nau-j, nau-b, nau-f, nau-i and nau-w, respectively. Each group was randomly divided into five subgroups of 5 mice each. *E. coli* cell cultures were diluted into five concentrations (Table II) with phosphate-buffered saline. *E. coli* cell culture (0.2 ml) of different concentrations was intraperitoneally injected into mice in the experimental subgroups, while 0.2 ml of sterile saline was intraperitoneally injected into each control mouse. The clinical performance including pneumorrhagia, renal edema and intestine wall thickness of all mice were observed once every 12 h for 7 days consecutively. Pathological changes were observed at necropsy and recorded. Liver, spleen, kidney and bladder smears were also observed under a light microscope to examine *E. coli* morphology. The present study was approved by the Animal Ethics Committee of the Nanjing Police Dog Research Institute of Ministry of Public Security (Nanjing, China). To minimize suffering, all surviving mice were sacrificed by decapitation, following the induction of anesthesia using 3% pentobarbital (Sigma-Aldrich; Merck KGaA) at dose of 200 mg/kg injected via the peritoneal cavity.

**Grouping of female dogs and hormone treatment.** A total of 33 healthy, nulliparous, crossbred female dogs (age, 2-3 years; weight, 8-12 kg) were purchased from Experimental animal center of Yangzhou University (Yangzhou, China). The experimental female dogs were divided into four groups, with 10 dogs in each of the three experimental groups and three dogs in the control group. Each dog was housed separately in the animal room and maintained in a 12-h light/dark cycle at ~20°C and 50-60% humidity with *ad libitum* access to commercial dog food and water during the experiments. After 2 weeks, all female dogs were mentally normal assessed with good activity (forceful and active limbs), normal temperature within the range of 38-39°C, with a normal eating action and good appetite, and a vaginal smear test was performed. The present research was approved by the Animal Ethics Committee of the Nanjing Police Dog Research Institute of Ministry of Public Security (Nanjing, China).

Prior to ovariectomy, general anesthesia was performed using the following anesthetics successively: Subcutaneous injection of 0.04 mg/kg atropine (Wuhu Kangqi Pharmaceuticals Co., Ltd, Wuhu, China) for pre-anesthesia; 10 min later, intramuscular injection of 0.05 ml/kg Sumianxin

Table I. Primers for the seven virulence genes.

Gene	Primer sequence (5'→3')	Annealing temperature, °C	Size of amplified fragments, bp
<i>hlyA</i>	F: AACAAAGGATAAGCACTGTTCTGGCT R: ACCATATAAGCGGTCATTCCCGTCA	63	1,177
<i>sfa</i>	F: CGGAGGAGTAATTACAAACCTGGCA R: CTCCGGAGAAGCTGGGTGCATCTTAC	63	410
<i>fim</i>	F: GAGAAGAGGTTTGATTTAACTTATTG R: AGAGCCGCTGTAGAAGCTGAGG	58	559
<i>pap</i>	F: GCAACAGCAACGCTGGTTGCATCAT R: AGAGAGAGCCACTCTTATACGGACA	63	336
<i>papC</i>	F: GACGGCTGTACTGCAGGGTGTGGCG R: ATATCCTTTCTGCAGGGATGCAATA	60	328
<i>papGIII</i>	F: CATGGCTGGTTGTTCCCTAAACAT R: TCCAGAGACTGTGCAGAAGGAC	63	421
<i>usp</i>	F: ATGCTACTGTTTCCGGGTAGTGTGT R: CATCATGTAGTCGGGGCGTAACAAT	66	1,000

F, forward; R, reverse.

Table II. Concentrations of five subgroups for each experimental group.

Group number	Strain	Concentrations, CFU
1	nau-h	7x10 <sup>7</sup> ; 4.67x10 <sup>7</sup> ; 3.10x10 <sup>7</sup> ; 2.07x10 <sup>7</sup> ; 1.38x10 <sup>7</sup>
2	nau-d	8x10 <sup>8</sup> ; 5.30x10 <sup>8</sup> ; 3.50x10 <sup>8</sup> ; 2.37x10 <sup>8</sup> ; 1.58x10 <sup>8</sup>
3	nau-o	7x10 <sup>7</sup> ; 3.50x10 <sup>7</sup> ; 1.75x10 <sup>7</sup> ; 8.75x10 <sup>6</sup> ; 4.38x10 <sup>6</sup>
4	nau-q	7x10 <sup>7</sup> ; 3.50x10 <sup>7</sup> ; 1.75x10 <sup>7</sup> ; 8.75x10 <sup>6</sup> ; 4.38x10 <sup>6</sup>
5	nau-j	1x10 <sup>8</sup> ; 6.67x10 <sup>8</sup> ; 4.45x10 <sup>8</sup> ; 2.96x10 <sup>8</sup> ; 1.98x10 <sup>8</sup>
6	nau-b	2.67x10 <sup>7</sup> ; 1.78x10 <sup>7</sup> ; 1.19x10 <sup>7</sup> ; 7.90x10 <sup>6</sup> ; 5.30x10 <sup>6</sup>
7	nau-f	2x10 <sup>8</sup> ; 1x10 <sup>8</sup> ; 5x10 <sup>7</sup> ; 2.50x10 <sup>7</sup> ; 1.25x10 <sup>7</sup>
8	nau-i	1x10 <sup>9</sup> ; 5x10 <sup>8</sup> ; 2.50x10 <sup>8</sup> ; 1.25x10 <sup>8</sup> ; 6.75x10 <sup>7</sup>
9	nau-w	1x10 <sup>8</sup> ; 5x10 <sup>8</sup> ; 2.50x10 <sup>8</sup> ; 1.25x10 <sup>8</sup> ; 6.25x10 <sup>8</sup>

CFU, colony-forming unit.

(Huamu Animal Health Products Co., Ltd., Changchun, China) as basal anesthesia, followed by intravenous injection of 4 mg/kg Zoletil (Virbac Laboratories, Carros, France) and isoflurane (Hebei Yipin Pharmaceutical Co., Ltd, Hebei, China) at the minimal alveolar concentration of 1.0%. Both ovaries of each dog were removed by a conventional laparotomy along

the linea alba. Analgesic (Meloxicam, Qingdao Orbiopharm Co., Ltd, Qingdao, China) was administered once daily continuously for 5 days following the ovariectomy at an initial dosage of 0.2 mg/kg and subsequent dose of 0.1 mg/kg. After 10 days, the stitches were removed.

After the female dogs had recovered from ovariectomy, estradiol and/or progesterone (Ningbo A second hormone factory, Ningbo, China) were administered 4 weeks later. Briefly, estradiol was administered to dogs in group 1 by intramuscular injection twice daily for 12 days (dose doubled every 3 days), with an initial dose of 0.6 µg/kg, followed by intramuscular injection of progesterone at 0.2-1.8 mg/kg, twice daily for 43 days. The progesterone was administered at an initial dose of 0.2 mg/kg and was increased by 0.2 mg/kg every three days, twice daily until the maximum of 1.8 mg/kg was reached after 24 days, and 1.8 mg/kg was maintained for a further 19 days. For dogs in group 2, only progesterone was administered by intramuscular injection at 1.8 mg/kg, twice daily for 55 continuous days. For dogs in group 3, only estradiol was administered by intramuscular injection with an initial dose of 0.6 mg/kg (dose doubled every 3 days for 12 days) and maintained at 4.8 mg/kg, administered twice daily for the following 43 days.

**Determination of estrous cycle.** The estrous cycle of female dogs was determined by observation of clinical symptoms, examination of changes in vaginal epithelial cells, routine blood tests and measurement of serum sex hormone levels.

Changes in the vaginal epithelial cells were examined by vaginal smear tests once every 1-2 days after hormone administration began until the proestrus of the simulated estrous cycle was observed, once a day during the estrus and dioestrus stages, and once every 3 days during the anestrus stage until the onset of disease. The keratinization extent of vaginal epithelial cells was evaluated by calculating superficial cell index (SCI), which was defined as the percentage of

superficial epithelial cells (including anuclear epithelial cells, epithelial cells and large interstitial cells) in total superficial cells and submerged epithelial cells (small interstitial cells and subsidiary basal cells). The first time when SCI was >80% was defined as the onset of estrus; an SCI value >80% with a small number of neutrophilic cells (NCs) was defined as staying in the estrus stage; a sharp decrease in SCI and a sudden appearance of a great many NCs occupied 70-80% of total cells in a microscopic field, which was defined as the onset of metestrus. Dogs were considered to be in the diestrus stage when SCI was <20% and NLs disappeared.

Meanwhile, blood samples were also collected every 5-7 days after the initiation of hormone administration until the experiments ended, each time at 2 h after hormone injection. A routine blood test was performed and serum estrogen and progesterone levels were measured. Canine abdominal B-scan ultrasound performed on a MyLab™ 30 VET (Esaote S.p.a., Trezzano S.N., Italy) was used to observe changes in size and shape of uterine horns until the experiments ended.

**Inoculation with *E. coli*.** According to the results of vaginal smear and blood sex hormone tests, when the dogs in group 1 were in the 12th day of dioestrus, inoculation with *E. coli* was performed in all three experimental groups. Before inoculation with *E. coli* into uterus, the enterocoelia of dogs were opened under general anesthesia, as follows: Subcutaneous injection of 0.04 mg/kg atropine (Wuhu Kangqi Pharmaceuticals Co., Ltd.) for pre-anesthesia; 10 min later, intramuscular injection of 0.05 ml/kg Sumianxin (Huamu Animal Health Products Co., Ltd.) as basal anesthesia, followed by intravenous injection of 4 mg/kg Zoletil (Virbac Laboratories) and isoflurane (Hebei Yipin Pharmaceutical Co., Ltd.) at the minimal alveolar concentration of 1.0%. After anesthesia, a venous indwelling needle was used to pierce the uterine wall of the right uterine horn, followed by injection of *E. coli* cultures at  $2 \times 10^7$  colony-forming units through a medical injector in the first three groups. After inoculation, the enterocoelia was closed via straticulate saturation of the abdominal wall muscles, subcutaneous tissue and skin, respectively. In each of the experimental groups, five dogs were injected with the strongly virulent *E. coli* strain, nau-b, and the other five dogs were injected with a weakly virulent strain, nau-i. Dogs in the control group were not inoculated with *E. coli*. After injection with different *E. coli* strains, the dogs were assessed for the successful induction of canine pyometra using the following criteria: When there was purulent eduction from vagina, it was diagnosed as the open-cervix pyometra. If numerous white blood cells and pus cells were detected by vaginal smear, in addition to the uterus augmentation to some extent were also detected, it was diagnosed as the closed-cervix pyometra.

**Histopathological observation of uterine tissues.** All dogs were euthanized using 3% sodium pentobarbital (90 mg/kg, Beijing Propbs Biotechnology Co., Ltd.) by intravenous injection, 80 days after *E. coli* inoculation. Afterwards, the uterus and the vaginal tissues were removed by laparotomy. A middle section of uterine horn was collected from each side and fixed in 10% neutral formaldehyde at 4°C for 24 h, followed by preparation into 5  $\mu$ m paraffin-embedded sections, which were subjected to dewaxing in xylene I and II, each for 10 min.

Subsequently, sections were stained with hematoxylin for 5 min at room temperature, stained with 0.5% eosin for 2 min, and treated with xylene twice, 5 min each time, and mounted.

Histopathological changes in uterine mucosa, stroma, glands and myometrium were observed under a microscope at x100 and x400 magnification. Endometrial thickness, myometrium thickness and uterine wall thickness were measured, and the ratio of endometrial thickness to myometrium thickness was calculated. The percentage of vesicle area in the total endometrium area was also calculated.

**Determination of ER and PR expression levels by reverse transcription-quantitative PCR (RT-qPCR).** RNA was extracted from the cultured uterus tissue cells using a RNAiso Plus kit (Takara Bio, Inc., Dalian, China) following the manufacturer's instructions. RNase-free Dnase I (Takara Bio., Inc.) was used to remove any genomic DNA contamination. RT was performed on ABI Prism 7300 Detection System (Applied Biosystems Inc. Carlsbad, CA, USA) in a 20  $\mu$ l reaction system using the PrimeScript RT Master Mix kit (Takara Bio, Inc.), which contained  $\sim 2 \mu$ g RNA template, 4  $\mu$ l buffer (5X), 2  $\mu$ l dNTP (10 mM), 1  $\mu$ l oligo (dT) 18 (10  $\mu$ M), 1  $\mu$ l RNase inhibitor, 1  $\mu$ l Rever-Tra Ace- $\alpha$  reverse transcriptase and 9  $\mu$ l RNase free dH<sub>2</sub>O. Subsequently, a 25  $\mu$ l PCR reaction system was used for DNA amplification, and the system contained 12.5  $\mu$ l 2xSYBR Green I master Mix (Takara Bio., Inc.) in addition to 1  $\mu$ l upstream and downstream primer each, 10  $\mu$ l cDNA and 0.5  $\mu$ l ddH<sub>2</sub>O. The reaction was conducted under the following conditions: Denaturation at 94°C for 4 min, followed by 20-32 cycles of denaturation at 94°C for 40 sec, annealing at 55°C for 40 sec, extension at 72°C for 40 sec and a final extension at 72°C for 7 min. The PCR products were examined by 1% agarose gel electrophoresis. The primers were as follows: GAPDH (as a control), forward 5'-AAAGCTGCC AAATATGACGA-3' and reverse 5'-TTTCGACGGTTTATA CTGCT-3'; ER, forward 5'-GACCCATCCAGACCCTTCA-3' and reverse 5'-CTGGGTAGGTCTGGGAAGT-3'; and PR, forward 5'-TAGCTCACAGCGTTTCTACCA-3' and reverse 5'-ATCGAGTGTGCAAAGATGGT-3'. mRNA expression levels were quantified using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (18), and the experiment was repeated three times.

**Statistical analysis.** All data were presented as the mean  $\pm$  standard error of the mean. Duncan's multiple analysis was performed by using SPSS, version 20.0 (IBM Corp., Armonk, NY, USA). GraphPad Prism version 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for multiple comparisons by ordinary one-way analysis of variance analyses followed by least-significant difference test as the post-hoc analysis. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Screening of *E. coli* strains.** Expression of 9 virulence genes (*hlyA*, *sfa*, *fim*, *pap*, *papC*, *icuD*, *cnfI*, *papGIII* and *usp*) among the 11 isolated *E. coli* strains was evaluated by PCR. Gene *hlyA* encoding hemolysin, *sfa* encoding S fimbriae, *pap* encoding P fimbriae, *papC* encoding P fimbriae and *papGIII* encoding adhesin were all expressed in 63.6% of the strains. The *fim*

Table III. Determination of LD<sub>50</sub> values in mice.

Strain	Number of bacteria, CFU	Mortality/subgroup (n=5 per group)	LD <sub>50</sub> , CFU
nau-b	2.67x10 <sup>7</sup> -5.3x10 <sup>6</sup>	5, 4, 3, 2, 0	1.0520x10 <sup>7</sup>
nau-i	1x10 <sup>9</sup> -6.25x10 <sup>7</sup>	5, 4, 3, 2, 0	2.0421x10 <sup>8</sup>
nau-h	7x10 <sup>7</sup> -1.38x10 <sup>7</sup>	5, 5, 3, 2, 0	2.5384x10 <sup>7</sup>
nau-d	8x10 <sup>8</sup> -1.58x10 <sup>8</sup>	5, 4, 2, 0, 0	4.0365x10 <sup>8</sup>
nau-o	7x10 <sup>7</sup> -4.375x10 <sup>6</sup>	5, 3, 1, 0, 0	2.8188x10 <sup>7</sup>
nau-q	7x10 <sup>7</sup> -4.375x10 <sup>6</sup>	5, 4, 3, 0, 0	1.9186x10 <sup>7</sup>
nau-j	6.67x10 <sup>8</sup> -1x10 <sup>8</sup>	5, 5, 4, 2, 0	3.3629x10 <sup>8</sup>
nau-f	2x10 <sup>8</sup> -1.25x10 <sup>7</sup>	5, 3, 1, 0, 0	8.0536x10 <sup>7</sup>
nau-w	6.25x10 <sup>8</sup> -1x10 <sup>8</sup>	5, 5, 3, 1, 0	2.0125x10 <sup>7</sup>
Control	0	0, 0, 0, 0, 0	-

LD<sub>50</sub>, median lethal dose; CFU, colony-forming unit.

Table IV. Expression of virulence genes in *Escherichia coli* strains, nau-b and nau-i.

Virulence gene	<i>Escherichia coli</i> strain	
	nau-b	nau-i
<i>sfa</i>	+	-
<i>hlyA</i>	+	-
<i>fim</i>	+	+
<i>pap</i>	+	-
<i>icuD</i>	-	-
<i>cnfI</i>	-	-
<i>papC</i>	+	-
<i>papGIII</i>	+	-
<i>usp</i>	+	-

gene encoding type I fimbriae was expressed in 90.9% of the strains and *usp* encoding a uropathogenic-specific protein was expressed in 54.5% of the strains.

Listlessness, loss of appetite and huddling in groups were observed in each test mouse 24 h after intraperitoneal injection of *E. coli* cultures. Mortality occurred in 114 mice within 24-72 h following inoculation (Table III) and mice that survived longer than 72 h were decapitated. Serous exudate was found at autopsy in the enterocoelia of all mice. Kidney swelling and uterine edema were observed in 96 mice. Gram-negative short, bacilliform bacteria were observed on the smears of liver, spleen, kidney and bladder of all mice that succumbed. No abnormalities were observed in the control mice.

Taking LD<sub>50</sub> values and the expression of virulence genes into consideration (Tables III and IV), a strongly virulent *E. coli* strain containing seven virulence genes (*hlyA*, *sfa*, *fim*, *pap*, *papC*, *papGIII* and *usp*), named nau-b (LD<sub>50</sub>; 2.0421x10<sup>8</sup>), and a weakly virulent strain containing *fim* only, named nau-i

(LD<sub>50</sub>; 1.0520x10<sup>7</sup>), were selected for later inoculation in female dogs.

#### Identification of estrous cycle and pyometra in female dogs.

The blood estrogen and progesterone levels changed with exogenous hormone levels in female dogs. According to the vaginal smear test and blood sex hormone test, a complete, simulated estrous cycle, including proestrus, estrus, metestrus, dioestrus and anestrus, was observed in the female dogs administered with both estradiol and progesterone. According to the status of the cervix, pyometra symptoms were observed at the end of dioestrus. In female dogs administered progesterone alone in group 2, were identified to remain in the dioestrus and were not observed to move into other estrous cycles, thus dioestrus was longer than for other groups, and pyometra symptoms were also observed at the end of the study. The onset time of pyometra in dogs in groups 1 and 2 inoculated with *E. coli* strain nau-b was on day 71±2.5, and that for the dogs inoculated with *E. coli* strain nau-i was on day 74±3.5. Except for one dog with closed-cervix pyometra in each group, the other dogs in groups 1 and 2 had open-cervix pyometra, with symptoms of purulent secretion from vagina and varying degrees of uterine enlargement according to the B-scan ultrasound. Swelling and congestion of uterine horns was demonstrated at autopsy in group 1 and 2 female dogs, with uterine horn diameter of 2-3.8 cm. Varying amounts of reddish-brown liquid with a strong odor were observed in the uterine cavity. Female dogs administered with estradiol alone were found in the proestrus stage until the end of experiments, and pyometra was not observed until autopsy. Pyometra was not observed in the control group.

The number of red blood cells and hemoglobin concentration were within the normal range in each group throughout the experiment, as were the levels of alanine aminotransferase, alkaline phosphatase, blood urea nitrogen, creatinine and transglutaminase. After *E. coli* inoculation, there was one dog found with a body temperature range of 39.3-39.6°C in each of groups 1 and 2, also with decreased appetite; however, neither dog stopped feeding completely.

#### Histopathological comparison of uterine horn among female dogs subjected to different treatments.

Abundant endometrial glands were observed in the uterine horn of female dogs inoculated with *E. coli* strain nau-b in group 1, as well as complete cystic endometrial hyperplasia (CEH) of the mucous membrane and superficial functional layer. The epithelium of some glands demonstrated papillary hyperplasia, forming labyrinth-like structures (Fig. 1A; magnification, x100). At magnification x400, the glandular epithelial cells were cylindrical and mucinous, containing abundant, bright cytoplasm, with nuclei located centrally. Mesenchyme was infiltrated by large numbers of inflammatory cells (Fig. 1B). Similar symptoms were observed in the uterine horns of group 1 dogs inoculated with strain nau-i, although CEH and infiltration of inflammatory cells was less severe.

At low magnification (x100), endometrial mucosa forming plica was observed in the uterine horn of female dogs inoculated with *E. coli* strain nau-b in group 2, with few cylindrical glands on the plica, and infiltration of focal

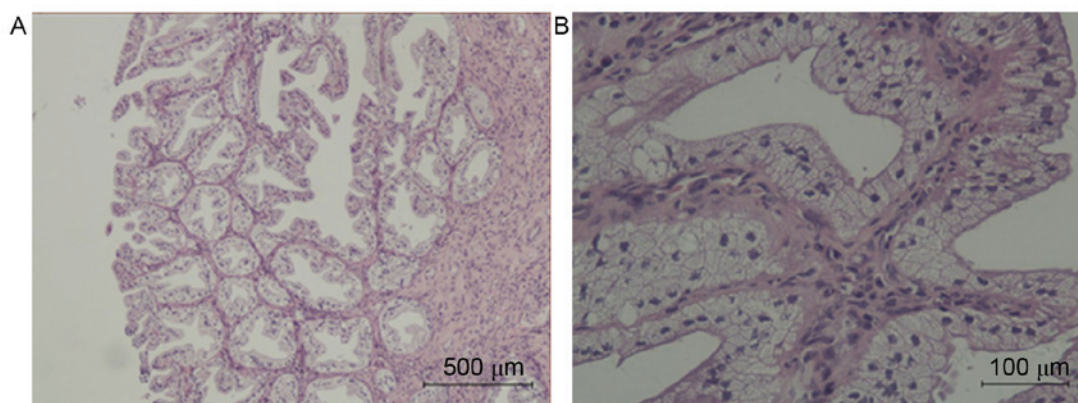


Figure 1. Histopathological observation of uterine tissues stained with hematoxylin and eosin in dogs treated with estradiol and progesterone. (A) Magnification, x100. Scale bar, 500  $\mu\text{m}$ . (B) Magnification, x400. Scale bar, 100  $\mu\text{m}$ .

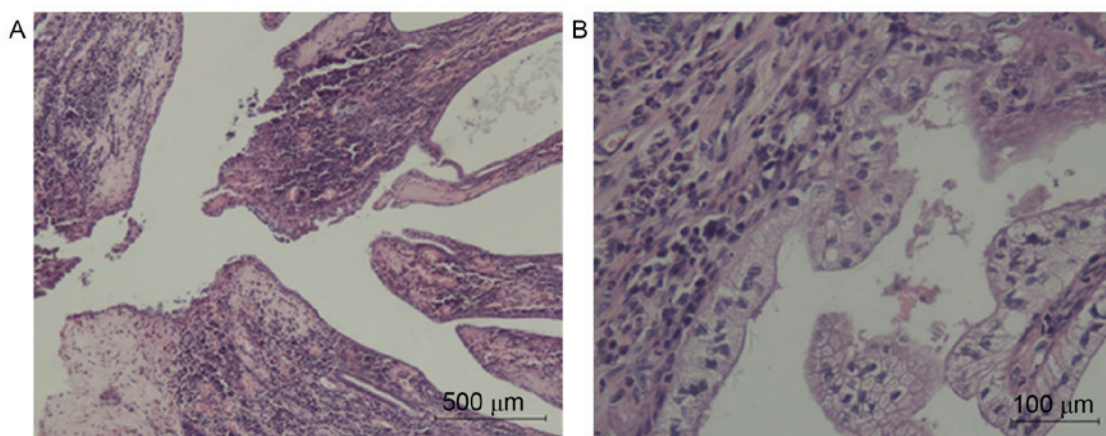


Figure 2. Histopathological observation of uterine tissues stained with hematoxylin and eosin in dogs treated with progesterone. (A) Magnification, x100. Scale bar, 500  $\mu\text{m}$ . (B) Magnification, x400. Scale bar, 100  $\mu\text{m}$ .

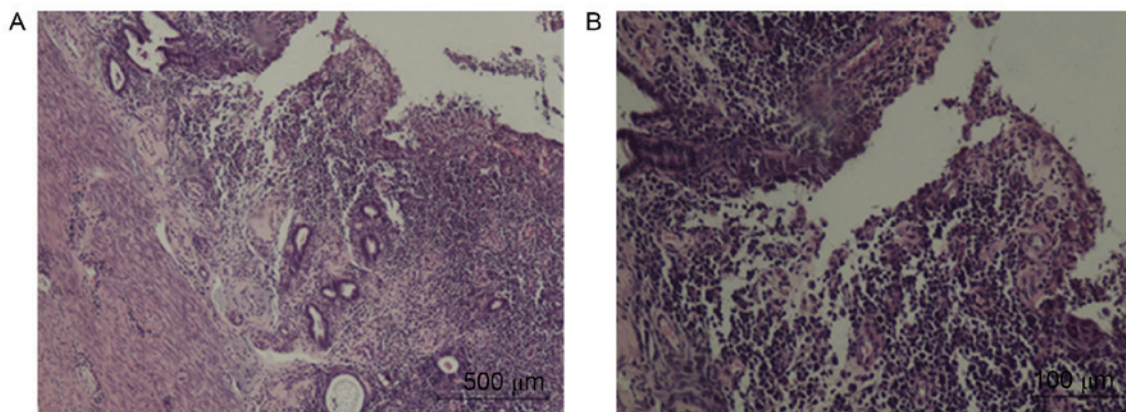


Figure 3. Histopathological observation of uterine tissues stained with hematoxylin and eosin in dogs treated with estradiol. (A) Magnification, x100. Scale bar, 500  $\mu\text{m}$ . (B) Magnification, x400. Scale bar, 100  $\mu\text{m}$ .

inflammatory cells (Fig. 2A). At magnification x400, the endometrial epithelial plicae had few glands, and CEH was observed, as well as infiltration by large numbers of plasma cells (Fig. 2B). Female dogs inoculated with nau-i in group 2 displayed similar symptoms at the uterine horns; however, with a less severe degree of CEH and infiltration of inflammatory cells.

The uterine horns of female dogs inoculated with *E. coli* strain nau-b in group 3 demonstrated fewer endometrial glands and exhibited no secretion by tubular glands or surface erosion; meanwhile myometrium was infiltrated by focal inflammatory cells (Fig. 3A; magnification, x100). The lumina of endometrial glands were observed to be small and the mesenchyme was infiltrated by inflammatory cells (Fig. 3B,

magnification, x400). Female dogs inoculated with nau-i in group 3 demonstrated similar symptoms at the uterine horns as those inoculated with nau-b.

In the uterine horn of female dogs in the control group at low magnification (x100), the surface of the uterine cavity had a single layer of columnar epithelium and exhibited proliferation of subepithelial fibrous tissue; no glands were observed; a thick mesenchyme was observed, and there was no obvious infiltration of inflammatory cells (Fig. 4A). A single layer of columnar epithelium contained a small number of stromal cells, no glands, infiltration by a small amount of lymphocytes and plasma cells, and a small amount of secretion in the uterine cavity (Fig. 4B; magnification, x400).

*Comparison of biometric indices among female dogs subjected to different treatments.* Endometrial thickness, myometrium thickness, uterine wall thickness and vacuole area were the largest in group 2 (with the exception of *E. coli* nau-i endometrial thickness; Table V), indicating that group 2, administrated with only progesterone was significantly more effective at promoting hyperplasia of the uterine wall than groups administrated with estradiol and progesterone in group 1 and only estradiol in group 3 (P<0.05). Furthermore, endometrial thickness, myometrium thickness and the thickness of the uterine wall in female dogs inoculated with *E. coli* nau-b were significantly increased compared with nau-i or the control group (P<0.05; Table V), indicating that *E. coli* nau-b induced significant hyperplasia of the uterine wall.

*Comparison of ER and PR expression levels in uterine horns.* ER expression levels in dogs inoculated with nau-i was the highest in group 2, followed by groups 1 and 3, respectively. In dogs inoculated with nau-b, the highest ER expression was exhibited in group 3, and this was significantly increased compared with the control (P<0.01; Fig. 5A). Furthermore, ER expression levels in dogs inoculated with *E. coli* nau-i in groups 1 and 2 were significantly higher compared with their counterparts in female dogs inoculated with nau-b (P<0.01 and P<0.05, respectively). In group 3, the ER expression level in dogs inoculated with *E. coli* nau-b was significantly higher than that in dogs inoculated with nau-i (P<0.05; Fig. 5A).

PR expression level in dogs inoculated with nau-i was the highest in group 1, followed by groups 2 and 3, respectively, with significant differences between the groups (P<0.01; Fig. 5B). PR expression level in dogs inoculated with nau-b was the highest in group 3, followed by groups 1 and 2, respectively. PR expression in dogs inoculated with nau-i was significantly higher in group 2 than group 3 (P<0.05; Fig. 5B). Furthermore, PR expression levels in dogs inoculated with *E. coli* nau-i in groups 1 and 2 were also significantly higher than those in their counterparts inoculated with nau-b (P<0.01; Fig. 5B). In group 3, the PR expression level in dogs inoculated with *E. coli* nau-b was significantly higher than that in dogs inoculated with nau-i (P<0.05; Fig. 5B).

**Discussion**

In the present study, pyometra was only found in female dogs administrated with progesterone alone or progesterone combined with estrogen, irrespective of the virulence of

Table V. Measurement of dog uterine parameters.

Parameter	Experimental group			
	Group 1	Group 2	Group 3	Control
	<i>E. coli</i> nau-b	<i>E. coli</i> nau-b	<i>E. coli</i> nau-b	<i>E. coli</i> nau-i
Endometrial thickness, mm	3.298±0.121 <sup>b-f</sup>	4.037±0.177 <sup>a,b,d,e,f</sup>	1.905±0.142 <sup>b,c,d,f</sup>	1.560±0.109 <sup>a-e</sup>
Myometrium thickness, mm	1.366±0.084 <sup>b-f</sup>	5.182±0.171 <sup>a,b,d,e,f</sup>	1.894±0.125 <sup>a,b,c,d,f</sup>	1.710±0.134 <sup>a,c,d,e</sup>
Uterine wall thickness, mm	4.746±0.146 <sup>b-f</sup>	9.298±0.302 <sup>a,b,d,e,f</sup>	4.061±0.099 <sup>a,c,d,f</sup>	3.520±0.178 <sup>a-e</sup>
Endometrial:myometrium thickness	2.422±0.185 <sup>b-f</sup>	0.780±0.031 <sup>a,b,e</sup>	1.011±0.114 <sup>a-d</sup>	0.913±0.097 <sup>a,b,d</sup>
Vesicle area, %	42.823±3.008 <sup>e-f</sup>	54.053±4.368 <sup>a,b,d,e,f</sup>	7.008±0.572 <sup>a-d</sup>	1.246±0.050 <sup>a-f</sup>

Data are expressed as the mean ± standard error of the mean. <sup>a</sup>P<0.05 nau-b subgroup in group 1 vs. all other groups, <sup>b</sup>P<0.05 nau-i subgroup in group 1 vs. all other groups, <sup>c</sup>P<0.05 nau-b subgroup in group 2 vs. all other groups, <sup>d</sup>P<0.05 nau-i subgroup in group 2 vs. all other groups, <sup>e</sup>P<0.05 nau-b subgroup in group 3 vs. all other groups, <sup>f</sup>P<0.05 nau-i subgroup in group 3 vs. all other groups.

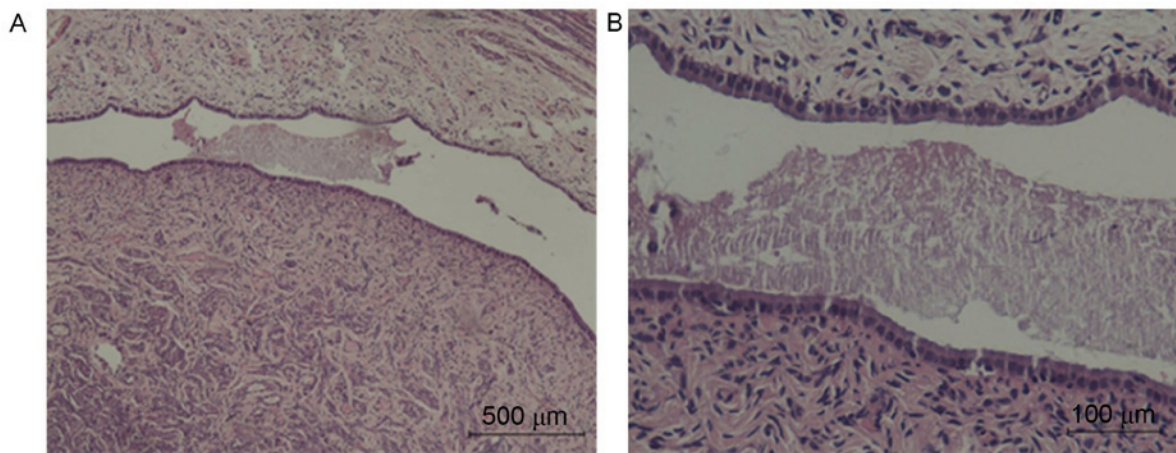


Figure 4. Histopathological observation of uterine tissues stained with hematoxylin and eosin in control dogs. (A) Magnification, x100. Scale bar, 500  $\mu\text{m}$ . (B) Magnification, x400. Scale bar, 100  $\mu\text{m}$ .

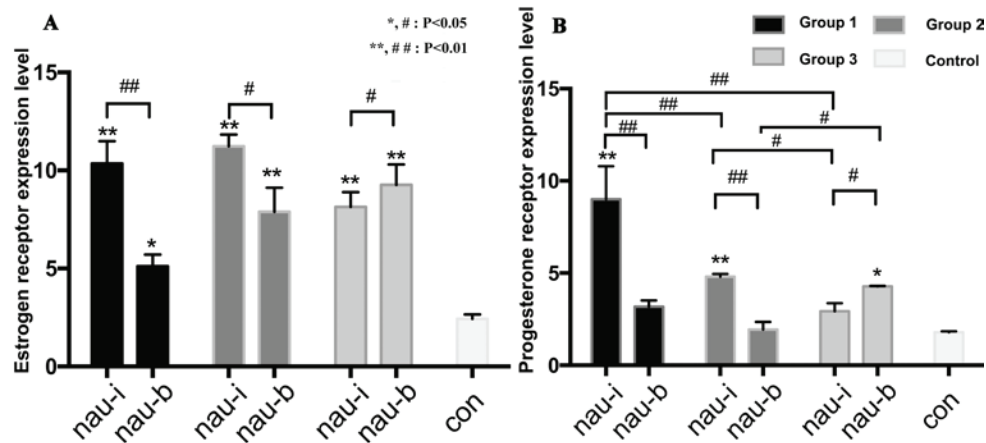


Figure 5. Ratio of estrogen and progesterone receptor expression levels in uterine horns of dogs in each group. (A) Estrogen receptor expression level. (B) Progesterone receptor expression level. Data are expressed as the mean  $\pm$  standard error of the mean. nau-i, weakly virulent *Escherichia coli* strain; nau-b, strongly virulent *Escherichia coli* strain. \* $P < 0.05$ , \*\* $P < 0.01$  vs. control, # $P < 0.05$ , ## $P < 0.01$  vs. corresponding group.

*E. coli*. This suggested that the occurrence of pyometra was closely related to the status of uteri in female dogs and progesterone had a more critical role in pyometra occurrence as compared with estrogen. This is consistent with previous studies (19,20). A study by Nomura *et al* (21) demonstrated that the success rate of inducing pyometra was highest when inoculating *E. coli* at the diestrus stage. Furthermore, plasma progesterone level is highest in the diestrus stage of the estrous cycle (22). Thus, inoculation of *E. coli* was performed on the 12th day of the diestrus stage of the simulated estrous cycle in the present study, when the progesterone was considered to be at a higher level.

Notably, clinical symptoms, histopathological observation and biometric data revealed more severe pyometra symptoms in female dogs administered with progesterone and estrogen as compared with those administered with progesterone alone, when the dogs were inoculated with the same *E. coli* strain. This may be attributed to the fact that estrogen enhances the stimulatory effects of progesterone on the uterus in female dogs administered with progesterone and estrogen (23). Furthermore, female dogs administered with both progesterone and estradiol demonstrated a higher PR level but lower

ER level compared with those administered with progesterone only when they were inoculated with the same *E. coli* strain. Thus, a higher PR level may lead to more severe symptoms in dogs with pyometra, whereas the opposite is the case for the ER level. This may be because progesterone causes more severe symptoms via binding to a larger number of receptors in dogs with pyometra.

In the present study, a strongly virulent *E. coli* strain, nau-b, containing seven virulence genes (*hlyA*, *sfa*, *fim*, *pap*, *papC*, *papGIII* and *usp*), and a weakly virulent strain, nau-i, containing *fim* only, were used for inoculation in female dogs. The clinical symptoms and histopathological observation indicated that strongly virulent *E. coli* strain, nau-b, caused earlier onset of pyometra symptoms and more severe pyometra symptoms than the weakly virulent *E. coli* strain, nau-i. This suggested that higher bacterial virulence may lead to more severe symptoms. This agrees with previous findings on the pathogenicity of *E. coli* (11,14,15,17), which was determined by its virulence genes encoding fimbriae, toxins and other factors. Furthermore, ER and PR levels in dogs inoculated with *E. coli* strain nau-i were higher than those inoculated with *E. coli* strain nau-b, indicating that strong bacterial



virulence is associated with lower receptor level. It is possible that strong bacterial virulence may inhibit the expression of hormone receptors; however, this has not been supported by any previous findings. Currently, views towards the changes in hormone receptor expression among different studies are controversial (9,10). In addition, the association between hormone receptor levels and the severity of pyometra has not been reported. Thus, it is difficult to determine the mechanism underlying lower expression of hormone receptor levels in female dogs inoculated with strongly virulent *E. coli* strains.

The results of the present study suggested that the occurrence of pyometra was closely related to the status of uteri in female dogs and progesterone had a more critical role in pyometra occurrence compared with estrogen. It was demonstrated that a higher PR level may lead to more severe symptoms in female dogs with pyometra, when the female dogs were inoculated with the same *E. coli* strain. It was also demonstrated that strongly-virulent *E. coli* may induce more severe pyometra symptoms than weakly-virulent *E. coli*, although PR and ER levels were lower in female dogs inoculated with strongly-virulent severity *E. coli*. In conclusion, the present study indicated that *E. coli* may affect the role of sex hormone receptors in pyometra. As there are few relevant studies currently available, the findings of the present study should be further validated.

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