

Aerobes and Levels of Estradiol and Progesterone in Cystic Endometrial Hyperplasia-Pyometra Complex Bitches

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Abstract: To determine which aerobic bacteria exert a marked effect on Cystic Endometrial Hyperplasia-Pyometra complex (CEH-P) and to determine from where these bacteria originate. About 48 privately owned bitches were diagnosed with CEH-P. Healthy vagina and uterine samples were obtained with cotton pledgets and cumulative uterine fluids from CEH-P cysts were collected with a syringe in utero to isolate and identify aerobic bacterial. A total of 99 isolates were identified and the primary aerobe in the CEH-P bitches was found to be *Escherichia coli* (33/99). The levels of estrone in bitches with endometritis and in bitches with pyometra and estrus were higher than the levels in diestrus bitches ($p < 0.01$). When ranked according to the levels of progesterone in a descending order (i.e., from high to low levels), the ranking was bitches with pyometra, bitches with endometritis, bitches in estrus and bitches in diestrus. These results suggest that in addition to common pathogenic bacteria differential in every area, casual bacterial contamination invariably occurred in bitches with CEH-P and the primary infection ascended from the vagina when the cervix was open. The researchers also inferred that both estrone and progesterone levels in CEH-P bitches were increased relative to the levels in healthy bitches and were even higher than that of the levels observed in estrus bitches. Estrone and progesterone pretreatment and uterine mucosa damage were interpreted as the preconditions for bacterial infection. The hormone levels in CEH-P bitches were similar to the endocrinal characteristics of peripartum bitches.

Key words: CEH-P, aerobes, progesterone and estrone levels, bitch, preconditions, China

INTRODUCTION

Pyometra is an emergent disease in both dogs and cats that causes dysfunction in animal reproduction and results in death due to septicemia and toxemia (Pretzer, 2008). Dogs with pyometra exhibit symptoms such as fever, polydipsia, vomiting, polyuria, abdominal distention, purulent vaginal discharge, tachycardia, tachypnea, rapid deterioration and sepsis (Wiebe and Howard, 2009). Due to its pathological characteristics (endometrial hyperplasia, adenomyosis, hemorrhage, purulence and mucometra or hydrometra) and its pathogenic characteristics, the disease is referred to as Cystic Endometrial Hyperplasia-Pyometra complex (CEH-P) (Ajadi *et al.*, 2008; Bigliardi *et al.*, 2004; Martinez-Jimenez *et al.*, 2009; Hagman *et al.*, 2006; Iglesias-Nunez *et al.*, 2008).

CEH-P is usually considered to be induced by pathological changes in the endometrium due to overexposure to progesterone by successive heat cycles and progesterone treatment (Niskanen and Thrusfield, 1998) and bacteria are considered to be the primary factors causing CEH-P (Pretzer, 2008; Smith, 2006).

However, very few studies of the relative estrone levels in dogs with CEH-P have been reported. The objective of this study was to determine which aerobic bacteria produce a marked effect on CEH-P pathology, the origin of these bacteria. It was also analyzed the relative progesterone and estrone concentrations in the blood of bitches with CEH-P.

MATERIALS AND METHODS

Cumulative fluids and serum from CEH-P bitches: This protocol was approved by Huazhong Agricultural University (Wuhan 430070, China). About 48 privately owned bitches were diagnosed with CEH-P including pyometra and endometritis. All bitches were treated by ovariohysterectomy. The diagnosis was based on case history and physical examinations and ultrasonography was applied to demonstrate an enlarged, fluid-filled uterus (Pretzer, 2008).

Further diagnosis of CEH-P was verified visually during the ovariohysterectomy and was validated by histopathological examination (Schlafer and Gifford, 2008). Cumulative fluids were collected with a syringe from

pyometra cysts *in vitro*. The samples were brought back to the laboratory for bacterial culture and identification.

Uterine secretions from healthy bitches: Uterine secretions were obtained from healthy bitches during sterilization for ovariohysterectomy. Uteri were brought to the laboratory for bacteria culture and identification.

Vaginal secretions from healthy bitches: Vaginal secretions were collected with sterilized cotton pledgets from healthy bitches. The pledget was immediately put into a sterilized test tube containing 1 mL physiological saline and was brought to the laboratory for bacteria culture and identification.

Sera from healthy and CEH-P bitches: About 32 sera samples were obtained for the measurement of progesterone and estrone concentrations. Sera from bitches with endometritis (13) and pyometra (9) were obtained from the above-mentioned patients and from among the nine healthy bitches, sera were obtained from bitches without any clinical disease in diestrus (5) and from bitches in estrus (4).

Culturing of isolated aerobes: The sample from uteruses and vaginas were cultured in nutrient broth. Broth opacity indicated aerobe growth. All samples were sectionally streaked on a blood plate, MacConkey Agar, Salmonella Shigella Agar and Eosin-methylene blue agar and were cultured at 37°C for 24 h. Mixed colonies were streaked again to isolate a pure culture. The characteristics such as size, opacity, shape, superficiality, altitude, fringe, burnish, hardness, smell, colorant and haemolyticus of every colony in each cultural medium were recorded in detail. Colonies were transferred into tubes containing culture medium and were cultured at 37°C for 18~24 h. Each strain was stored at 4°C for later biochemical identification.

Identification of aerobes: Colonies from the stored tubes were rejuvenated and proliferated in nutrient broth at 37°C for 8 h. Each strain was smeared and dyed with gram staining. Strains were initially categorized as gram-positive coccus and bacilli or gram-negative coccus and bacilli based on their chromaticity, shape, size and alignment. Bacteria were transferred onto solid media and were cultured aerobically at 37°C for 18~24 h.

For further identification, cocci were cultured aerobically at 37°C for 24 h in Staphylococci biochemical assessor, O-F microdosis assessor, sucrose assessor, lactose assessor, sorbin assessor, amylo maltose assessor and indole assessor. The culture media and conditions for the bacilli were the same for cocci except

Enterobacteriaceae assessor was used instead of the Staphylococci biochemical assessor. Identified results were recorded in detail.

Pathological model of CEH-P with aerobes: About 6 healthy hybrid bitches were randomly divided into 3 groups and each group contained three bitches. All of the bitches were injected subcutaneously with diethylstilbestrol. Aerobe broth (5 mL, 2×10^5 CFU mL⁻¹) was introduced into their uteruses with a catheter and a syringe in the trial group three times every 2 days. The control animals were inoculated with broth without bacteria. Body temperature, vaginal secretion characteristics, vaginal mucosa characteristics and uterine ultrasonography results were recorded in detail for 1 month.

Pathogenicity of aerobes in mice: About 54 mice were randomly divided into 8 groups. Aerobe broth (1 mL, 2×10^5 CFU mL⁻¹) made from bacteria isolated from bitches with pyometra was introduced into the abdominal cavities of the mice. Broths with the following aerobes were used (one aerobe per group of mice): *Escherichia coli*, *Salmonella enterica*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus agalactiae*, Shigella and Corynebacteria. The group injected with broth without any bacteria served as a control. The experiment lasted 5 days and the morbidity of the mice was recorded.

Analysis of progesterone and estrone concentrations in sera: An iodine [¹²⁵I] Progesterone radioimmunoassay kit and an iodine [¹²⁵I] Estradiol radioimmunoassay kit were used to measure the progesterone and estradiol concentrations in sera. SPSS 13.0 was used for data analysis.

RESULTS

The number of species and strains of aerobic bacteria: No bacteria were isolated from healthy uteruses. All of the aerobes isolated from vaginas and from the uteruses of bitches with CEH-P are shown in Table 1. About 8 aerobes (99 colonies) were isolated and identified. The most frequent aerobe was *Escherichia coli* (33/99); *Salmonella enterica* (14/99), *Proteus mirabilis* (13/99), *Pseudomonas aeruginosa* (13/99), *Staphylococcus aureus* (12/99), *Streptococcus agalactiae* (7/99), Shigella (6/99) and Corynebacteria (1/99) were also found.

Aerobes in bitches with CEH-P: The aerobes isolated from the CEH-P contents are shown in Table 2. About 8 aerobes species were identified from the 87 colonies that

Table 1: The results of microscopic examination and biochemical identification of the aerobes

| Bacteria | <i>Escherichia coli</i> | <i>Salmonella enterica</i> | <i>Proteus mirabilis</i> | <i>Pseudomonas aeruginosa</i> | <i>Staphylococcus aureus</i> | <i>Streptococcus agalactiae</i> | Shigella | Corynebacteria |
|------------------------------|--|---|---|---|---|---|---|---|
| Colony amount | 33 | 14 | 13 | 13 | 12 | 7 | 6 | 1 |
| G ⁺ | G ⁻ | G ⁻ | G ⁻ | G ⁻ | G ⁺ | G ⁺ | G ⁻ | G ⁺ |
| Haemolyticus | +/- | - | - | + | + | + | - | - |
| O/F test | Fermentation | Fermentation | Fermentation | Fermentation | Fermentation | Fermentation | Fermentation | Fermentation |
| Triple sugar iron | bevel acid (+) bevel acid(+) bevel acid (+) aerosis (+) | bevel alkali (+) bevel acid (+) bevel acid (+) aerosis (+) | bevel acid (+) alkali (+) aerosis (+) | bevel alkali (+) bevel acid and alkali (+) aerosis (-) | bevel acid (+) bevel acid (+) aerosis (-) | bevel acid (+) bevel acid (+) aerosis (-) | bevel alkali (+) bevel acid (+) aerosis (-) | bevel acid (+) bevel acid (+) aerosis (+) |
| Benzopyrrole test | H ₂ S (-) | H ₂ S (-) | H ₂ S (+) | H ₂ S (+) | H ₂ S (-) | H ₂ S (-) | H ₂ S (-) | H ₂ S (-) |
| Methyl red test | + | - | -/+ | - | - | - | - | + |
| VP test | + | + | + | + | + | +/- | + | + |
| Gelatin test | - | - | - | - | - | - | - | - |
| Esculin | - | - | -/+ | + | - | -/+ | + | -/+ |
| 40% bile | -/+ | - | - | - | + | + | + | + |
| Citral test citric acid test | - | - | -/+ | + | - | - | - | - |
| Sorbite | + | - | - | - | - | - | + | + |
| Manicol | + | - | +/- | - | +/- | -/+ | + | + |
| Glucose | + | - | + | + | + | + | + | + |
| Lactose | + | - | +/- | - | - | +/- | - | + |
| Sucrose | + | - | +/- | - | -/+ | -/+ | - | + |
| Urea | - | - | -/+ | + | - | -/+ | - | + |

+positive, -negative, +/-many strains positive, -/+many strains negative

Table 2: The identification results for the aerobes isolated from pyometra contents

| Bacterium | Pyometra contents | | Vaginal secretion | | Total | |
|---------------------------------|-------------------|---------|-------------------|---------|---------------|---------|
| | Colony amount | Percent | Colony amount | Percent | Colony amount | Percent |
| <i>Escherichia coli</i> | 30 | 30.3 | 3 | 3.0 | 33 | 33.0 |
| <i>Salmonella enterica</i> | 11 | 11.1 | 3 | 3.0 | 14 | 14.1 |
| <i>Proteus mirabilis</i> | 11 | 11.1 | 2 | 2.0 | 13 | 13.1 |
| <i>Pseudomonas aeruginosa</i> | 12 | 12.1 | 1 | 1.0 | 13 | 13.1 |
| <i>Staphylococcus aureus</i> | 10 | 11.1 | 2 | 2.0 | 12 | 13.1 |
| <i>Streptococcus agalactiae</i> | 7 | 7.0 | - | - | 7 | 7.0 |
| Shigella | 5 | 5.0 | 1 | 1.0 | 6 | 6.0 |
| Corynebacterium | 1 | 1.0 | - | - | 1 | 1.0 |
| Total | 87 | 87.8 | 12 | 12.2 | 99 | 100.0 |

were isolated from bitches with CEH-P. The aerobes were the same as those listed above. The total number of colonies was 87 (87/99) and the most frequent bacterium was *E. coli*.

Aerobes in vaginas: About 6 aerobes (*Escherichia coli*, *Salmonella enterica*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and Shigella) were isolated from vaginal secretions in healthy dogs (Table 2). Researchers did not detect *Streptococcus agalactiae* or Corynebacteria and the number of colonies was low (12/99).

Pathogenicity of aerobes in bitches: The bitches treated with diethylstilbestrol and bacteria exhibited vaginal endomembrane hyperemia, estus and yellow mucous secretions. However, they recovered 3 days later. The bitches without treatment only exhibited vaginal endomembrane hyperemia and estus and they recovered on the 2nd day. None of the bitches showed any abnormality after 1 month.

Pathogenicity of aerobes in mice: Mice inoculated with bacteria started to die on the 2nd day; their morbidity and time of death are shown in Table 3. All of the mice treated with *E. coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* died on the 2nd day; all of the mice treated with *Salmonella enterica* died on the 3rd day. The frequencies of mortality in the other groups ranged from 66.7-88.3%. There was no death in the control group. The bacteria isolated from the ascites of dead mice were the same as those from the inoculation.

Estradiol and progesterone concentrations in sera: The levels of estrone in bitches with endometritis, bitches with pyometra and bitches in estrus were higher than that in diestrus bitches (p<0.01). In addition, the level of estrone was higher in pyometra bitches than in estrus bitches (p<0.05). The estrone concentration in endometritis bitches was higher than that in estrus bitches (p<0.01). No significant difference in the estradiol levels were found between endometritis and pyometra bitches. Progesterone concentrations were different among all 4 groups (p<0.01)

Table 3: The morbidities of mice treated with different bacteria

| Bacterium | No. of deaths at 12 h | No. of deaths at 24 h | No. of deaths at 48 h | No. of deaths at 72 h | No. of deaths at 96 h | Total no. of deaths | Morbidity (%) |
|---------------------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|---------------------|---------------|
| <i>Staphylococcus aureus</i> | 0 | 2 | 4 | 0 | 0 | 6 | 100.0 |
| <i>Escherichia coli</i> | 0 | 4 | 2 | 0 | 0 | 6 | 100.0 |
| <i>Pseudomonas aeruginosa</i> | 3 | 2 | 1 | 0 | 0 | 6 | 100.0 |
| <i>Salmonella enterica</i> | 0 | 0 | 4 | 2 | 0 | 6 | 100.0 |
| <i>Streptococcus agalactiae</i> | 0 | 3 | 2 | 0 | 0 | 5 | 83.3 |
| Shigella | 0 | 3 | 2 | 0 | 0 | 5 | 83.3 |
| Corynebacteria | 0 | 4 | 0 | 0 | 0 | 4 | 66.7 |
| <i>Proteus mirabilis</i> | 0 | 4 | 0 | 0 | 0 | 4 | 66.7 |
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 0.0 |

Table 4: The levels of estrone and progesterone in the blood

| Groups | Samples | Estrone concentration (pg mL ⁻¹) | Progesterone concentration (ng mL ⁻¹) |
|------------------|---------|--|---|
| Diestrus bitches | 5 | 34.77±14.52 ^A | 0.52±0.29 ^A |
| Estrus bitches | 4 | 535.37±213.24 ^B | 4.94±0.24 ^B |
| Endometritis | 13 | 1818.55±745.10 ^C | 9.53±3.31 ^C |
| Pyometra | 9 | 1318.89±619.74 ^{BC} | 641.00±330.00 ^D |

The same letter in a column indicates no difference (p>0.05); a different lower case letter indicates a significant difference at p<0.05; a different upper case letter indicates significant difference at p<0.01

in a descending order of progesterone levels, from high to low, the groups were pyometra, endometritis, estrus and diestrus bitches (Table 4).

DISCUSSION

Aerobic pathogens: Certainly, bacteria were a direct factor in bitches developing CEH-P (Hagman *et al.*, 2006; Hagman and Kuhn, 2002; Pretzer, 2008; Smith, 2006; Siqueira *et al.*, 2009). Although, the most common bacterium in bitches with CEH-P was *E. coli* (Dhaliwal *et al.*, 1998; Fransson *et al.*, 1997; Siemieniuch *et al.*, 2005), other anaerobic and aerobic bacteria such as *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Streptococcus canis*, *coagulase-negative Staphylococcus* sp., *Enterobacter aerogenes*, *Streptococcus dysgalactiae* ssp., *equisimilis*, *Proteus mirabilis*, *Serratia marcescens*, *Staphylococcus intermedius* and *Pseudomonas aeruginosa* were also detected in bitches with CEH-P (Gibson, 1998; Lee *et al.*, 2006; Zdunczyk *et al.*, 2006).

Moreover, *Citrobacter* sp. was isolated from canines with pyometra and hydrometra (Lee *et al.*, 2006). Even *Clostridium perfringens* was isolated from the uterine lumen of a bitch with emphysematous pyometra (Hernandez *et al.*, 2003).

The results indicated that the primary aerobes in most cases were similar but the dogs living at different sites could be infected by diverse bacteria. Bacteria were isolated from all of the bitches with vaginitis and the aerobes were *E. coli*, *Streptococcus canis*, beta-hemolytic *Streptococcus*, *Staphylococcus aureus*, *Staphylococcus intermedius*, *Proteus mirabilis* and *Pasteurella multocida* (Zdunczyk *et al.*, 2006; Kustritz, 2006;

Watts *et al.*, 1996, 1997, 1998). Intrauterine bacteria that ascended from the vagina during proestrus and estrus induced cystic endometrial hyperplasia/ pyometra in bitches during metestrus (Noakes *et al.*, 2001). Nevertheless, more information was necessary to explain the relationship between the bacterium species found in bitches with CEH-P in healthy vaginas and in the uterus.

To determine the source of the bacteria, it was compared aerobes from bitches with CEH-P, bitches with vaginitis and bitches with healthy vaginas and uteruses. Researchers did not isolate any aerobes from healthy uteruses and the bacterial species in the uteruses of bitches with CEH-P were mostly like those in the vagina. *Escherichia coli*, *Salmonella enterica*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus agalactiae*, Shigella and Corynebacteria were isolated from bitches with CEH-P and *Escherichia coli*, *Salmonella enterica*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and Shigella could be identified in healthy vaginas. Although, *Streptococcus agalactiae* and Corynebacteria were not found in the vagina, they accounted for 8% of the total isolates. These results suggested that the aerobes found in the uteruses of bitches with CEH-P ascended from the vagina and that the aerobes in vagina came from environmental contamination. Likewise, contamination of different sites in bitches with CEH-P occurred passively. The viewpoint could be also supported by other infectious diseases in animals.

The aerobes found in soft tissue abscesses, mastitis, pericarditis, pyometra and joint and foot abscesses in cattle, sheep and goats include *Microaerophilic coccus*, *Corynebacterium pyogenes*, *Fusobacterium necrophorum*, *Peptostreptococcus indolicus* and *Bacteroides* sp. (Slee, 1985). However, the above-mentioned bacteria were isolated less frequently in healthy cows than in cows with pyometra and cows with retained fetal membranes.

In cows with pyometra and retained fetal membranes, coliform were only found in the early stages of disease and the most common bacteria were *Corynebacterium pyogenes*, *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* (Olson *et al.*, 1984). These results

indicated that the bacteria species in affected uteruses are related to those in the vagina and other body places.

Progesterone and estrone: CEH-P is typically secondary to pathological changes that are induced in the endometrium due to overexposure to progesterone in successive heat cycles (Noakes *et al.*, 2001; Hardy and Osborne, 1974). The hypothesized pathogenic mechanism was that bacteria ascended into the uterus while the cervix was open because of estrone during proestrus and estrus (Sandholm *et al.*, 1975; Noakes *et al.*, 2001; Versteegen *et al.*, 2008). After that time, progesterone causes endometrial hyperplasia and luminal fluid accumulation, suppresses leukocyte activity and decreases myometrial activity. Reduced contractility and fluid accumulation favor ascending bacterial infections which are typically due to enteric bacteria from the vagina (Lee *et al.*, 2000; Noakes *et al.*, 2001; Smith, 2006). Analysis of progesterone receptor expression supports the above hypothesis (De Cock *et al.*, 1997; Noakes *et al.*, 2001; Sugiura *et al.*, 2004; Ververidis *et al.*, 2004). The significance of uterine trauma to pyometra formation was also confirmed (Noakes *et al.*, 2001).

A negative correlation has been demonstrated between progesterone and estrone receptor expression within both the canine CEH-P and control groups. Generally, progesterone was the main uterine receptor regulator for both the progesterone and estrone receptors during diestrus and early anestrus in healthy and affected uteruses. High progesterone levels suppress the expression of the progesterone and estrone receptors. It has been shown that the estrone receptor is expressed at a low level in the squamous metaplastic epithelium in the uterus of dogs with CEH-P (Ververidis *et al.*, 2004). However, the same expression was also found in the columnar epithelium during the normal estrus cycle of dogs (De Cock *et al.*, 1997). As for progesterone, it was proposed that the level of estrone was also increased in CEH-P bitches.

Although, the receptor expression has not been analyzed, the stronger hormone roles of progesterone and estrone could be argued. The amount and strength of a receptor is usually regulated depending on the functional demand of the animal and the expression can be directly adjusted by the concentration of regulators and by the response level.

It has been shown that progesterone and estrone levels increased in peripartum women and those who delayed to the 3rd day after delivery (Klier *et al.*, 2007). The results obviously showed that progesterone levels increased in bitches with CEH-P and were even higher than those in estrus bitches. In particular, estrone levels

in CEH-P bitches were higher than in estrus bitches. These hormone levels in CEH-P bitches should be similar to the endocrinal characteristics of peripartum bitches. The signaling pathways in both normal and pathological conditions should be investigated.

CONCLUSION

In this study, the results support the hypothesis; the most frequent aerobic pathogens in the uteruses of bitches with CEH-P ascended from the vagina and bacteria in healthy vaginas came from the environment. Additionally, both progesterone and estrone levels were elevated in bitches with CEH-P and these hormone levels in CEH-P bitches could be similar to the endocrinal characteristics of peripartum bitches.

In addition, researchers hypothesize that estrone and progesterone pretreatment and uterine mucosa damage were preconditions of the bacterial infections.

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