PhD THESIS

Laboratory diagnostic of mammary gland inflammations in lactating bitches

[SUMMARY OF THE Ph.D. THESIS]

Ph.D. student Iosif Vasiu

Scientific coordinator Prof.univ.dr. Florinel Gheorghe Brudășcă
INTRODUCTION

The uniqueness of mammals comes from the presence of the lactating mammary glands (*glandulae mammariae*), thus being the only animals with such features (KÖNIG and LIECBICH, 2007), which grant its taxonomy (SIMIONESCU, 1983).

In comparison with bitches, in dairy cows mastitis is very extensively studied. In bitch, even though oncology aspects are well studied (RUTTEMAN and KIRPENSTEIJN, 2003) in comparison with human breast cancer (OWEN, 1979) and even if the inflammed mammary gland is considered to be an emergency (ALDRIDGE and O’DWYER, 2013; MARTINS-BESSA et al., 2015), most of the time the condition is being overlooked and the literature regarding this matter is very scarces.

The literature survey included in the present study on bitch mastitis comprises an experimental model (VERVERIDIS et al., 2007), a study regarding presence of lactobacilli in bitch milk (MARTÍN, 2009), papers that look into puppy mortality secondary to mastitis (SCHÄFER-SOMI et al., 2003; MILANI et al., 2012), bitch mastitis case reports (HASEGAWA et al., 1992; LEE et al., 2009; MARINA ARAÚJO et al., 2011; MURAI et al., 2013; SIDNEI NUNES de OLIVEIRA et al., 2015) as well as a paper which looks into different laboratory changes in bitches with inflammed mammary glands (DZIECIOŁ et al., 2006). Quite recently, research has also been carried out regarding aerobic bacterial strains from dams with mastitis (TOYDEMIR et al., 2015).

In spite of the importance of subclinical mastitis in causing mortality of puppies, thorough studies are scarce (CAROLA JUNG et al., 2002; SCHÄFER-SOMI et al., 2003). This study aims to evaluate mammary gland inflammation dynamics, as well as subclinical mastitis impact on canine reproduction in both periparturient and *Lactatio sine graviditate* affected bitches.

Key words: bitch, diagnosis, lactation, mastitis
STRUCTURE OF THE THESIS

The present study comprises 143 pages, divided in two parts. The first part, literature study, consists of 7 chapters (49 pages) where histologic, anatomical and physiological aspects of canine mammary gland as well as data regarding milk biochemical and biophysical components are mentioned. In this section, milk bacterial strains and canine haematological and biochemical aspects are also displayed.

The second part (94 pages) is comprised in 9 distinct chapters, covering personal obtained data during a four year period (2012-2016). In this section, data regarding aims of research, working methodology, followed by in subsequent chapters by individual descriptions of research design introductory data on the main objectives, materials and methods and results displayed in a chronological manner followed by discussion section where obtained data were compared with pre exiting literature data. In the final conclusion section most salient points where highlighted. Main part of results from this section of the thesis are displayed in 27 tables and annexes, and 50 figures and pictures.

RESULTS OF THE RESEARCH

A total of 100 bitches (Canis lupus familiaris L.) (Annex 1) between 10 and 168 months of age (mean 36 months) (Annex 1), the majority of them being adults (76%) (Fig. 1), large (51%) and medium size (30%) breeds (Fig. 2), were included in this study.

From these bitches, 186 milk samples were collected, the majority from spring (29%), summer (26%) and autumn (25%). The least samples were collected during winter (20%) (Fig. 11).

From postpartum dams, 79% of milk samples were collected and only 4% from antepartum bitches. The remaining 17% of samples were collected from Lactatio sine graviditate bitches (Fig. 12).

From the collected samples, 11% were from hardened mammary glands, 5% from mammary glands with modified mammary secretions, 3% from hardened modified mammary secretion mammary glands and 1% from inflammed mammary glands and from mammary glands with tumours, each (Fig. 13).

Milk samples were manually collected in sterile vials (Nunc TM, Waltham, USA), varying from a few drops to 4 ml of milk, by using sterile gloves after local disinfection of the mammae with 70% sanitary alcohol as well as after clipping local hair (MARTÍN şi colab, 2010), and immediately transported to the Infectious Disease Department at the University of Agricultural Science and Veterinary Medicine Cluj-Napoca, for immediate testing.

In chapter 10, "Bitch milk cytology examination ", a total of 157 milk samples (between 0.5 - 4 ml for individual sample) were collected from 89 lactating bitches (4% were in the ante-partum period, 80% were in the post-partum period and 16% were females with Lactatio sine graviditate), belonging to 31 different breeds, parity, ages and body sizes. From the collected samples (i.e. 157), 7 (4%) were from the ante-partum period, 127 (81%) where from the post-partum period and 23 (15%) from
bitches with *Lactatio sine graviditate*. For the ante-partum period, milk was collected in the last week before parturition, whilst for the post-partum period milk was harvested between the first and the 6th week post-partum. Because it was very difficult for owners to estimate when galactorrhea started, for dams with *Lactatio sine graviditate* this data was not recorded.

Samples were centrifuged for 10 minutes at 2500 rpm/min. After centrifugation, from the obtained sediments, milk smears were obtained by using the “squash” and MGG (E. Merk, Darmstadt, Germany) techniques (MEYER et al., 2010). Six (i.e. 6) of the samples could not be interpreted due to dye flaws.

Clinical healthy mammary glands were characterized by the presence of a moderate to a high number of somatic cells, accompanied by high levels of cellular debris, scares too many squamous epithelial cells, few neutrophils, macrophages, erythrocytes and foamy cells. Presence of bacteria and phagocytosis has also been identified in isolated cases with no clinical relevance.

In dams with congested mammary glands, small numbers of somatic cells, accompanied by increased numbers of cellular debris, few neutrophils, inactivated macrophages and epithelial cells were spotted on milk smears.

In subclinical mastitis, somatic cells were moderate to high, accompanied by slightly elevated numbers of degenerated neutrophils, many foamy and epithelial cells along with activated macrophages, bacteria and phagocytosis. Scattered cellular debris, erythrocytes and eosinophils were also encountered.

Retention mastitis episodes were characterized by a moderate to a high number of somatic cells. On milk smears, epithelial cells, eosinophils, activated macrophages, foamy cells and degenerated neutrophils accompanied by bacteria and phagocytosis, were the dominant features. Cellular debris and erythrocytes had also been identified.

*Mastitis acuta* (including *Mastitis gangrenosa*) episodes were characterized by an increased number of foamy cells, degenerated neutrophils, cellular debris and bacteria accompanied by phagocytosis. The presence of small numbers of activated macrophages, eosinophils and erythrocytes were also encountered.

In the ante-partum period 57.14% (4/7, CI 95%: 18.41-90.10) of the tested samples were from healthy mammary glands, 28.57% (2/7, CI 95%: 3.67-70.96) were diagnosed with *Mastitis acuta* whilst 14.29% (1/7, CI 95%: 0.36-57.87) of the tested mammary glands were with retention mastitis (Fig. 34).

From the post-partum period, 51.97% (66/127, CI 95%: 42.93%-60.91%) of the tested mammary glands were clinically healthy, 22.05% (28/127, CI 95%: 15.18-30.26) were diagnosed with subclinical mastitis, 13.39% (17/127, CI 95%: 8.00-20.56) were diagnosed with *Mastitis acuta*, 4.72% (6/127, CI 95%: 1.75-10.00) were diagnosed with retention mastitis, 3.94% (5/127, CI 95%: 1.29-8.95) were diagnosed with mammary congestion, and only 0.79% (1/127, CI 95%: 0.02-4.31) of the tested mammary glands were diagnosed with *Mastitis gangrenosa* (Fig. 34).

From dams with *Lactatio sine graviditate*, 65.22% (15/23, CI 95%: 42.7-83.63) were diagnosed with retention mastitis, 13.04% (3/23, CI 95%: 2.78-33.59) were diagnosed with *Mastitis acuta*, 4.35% (1/23, CI 95%: 0.11-21.95) were diagnosed with subclinical mastitis and only 8.70% (2/23, CI 95%: 1.07-28.04) of the tested mammary glands were clinically healthy (Fig. 34). In bitch, the lactation period holds great influence over the different types of mammary gland inflammations (p<0.05).
In chapter 11, "Bitch milk pH evaluation", lactating bitch milk pH was evaluated, including samples from Lactatio sine graviditate bitches and from each individual type of mastitis encountered.

In order to evaluate milk pH in lactating bitches, 160 (86%) milk samples were evaluated by using litmus paper (E. Merk, Darmstadt, Germany) (HASEGAWA et al., 1992; DZIECIOŁ et al., 2006). One drop of milk was taken with a sterile microbiological loop, and based on colour reaction, milk pH was interpreted.

From the antepartum period, Mastitis acuta mammary glands presented an acidic milk pH (2/2, IC 95% 100). In this period, there was only one mammary gland diagnosed with subclinical mastitis, gland which had and alkaline milk pH value. Healthy mammary glands showed an acidic milk pH reaction (4/4, IC 95% 100).

In the postpartum period, Mastitis acuta mammary glands had in 75% (9/12, IC 95% 42.81-94.51) of cases alkaline milk pH. Healthy mammary glands had an acidic milk pH in 73.02% (46/63, IC 95% 60.35-83.43) of the tested milk samples. Glands with subclinical mastitis had an alkaline milk pH value in 80.77% (21/26, IC 95% 60.65-93.45) of tested samples. Congested mammary glands had an acidic milk pH reaction in 80% (4/5, IC 95% 28.36-99.49) of the cases, followed by an alkaline reaction in 20% (1/5, IC 95% 0.51-71.64) of cases. Milk samples from retention mastitis samples had an alkaline pH reaction in 83.33 % (5/6, IC 95% 35.88-99.58) of tested samples (Tabel 9).

Milk samples from Lactatio sine graviditate bitches posed an alkaline milk pH reaction in 100% (15/15, IC 95% 100.00) of tested samples.

In chapter 12, "Aerobic microbiome carriage in lactating bitch mammary gland", milk aerobic bacterial strains were evaluated from both periparturient and Lactatio sine graviditate bitches, highlighting the role of this strains in the genesis of mammary gland inflammations.

In order to evaluate bitch milk bacterial strains carriage, 184 milk samples were evaluated from all mammary glands (Fig. 10). Standard microbiological strains were used (Oxoid Limited, Hampshire, UK) (QUINN şi colab., 1994). For Bacillus identification, commercial API 50 CH (BioMérieux, l’Étoile, France) tests were used, whereas for G+ and G- strains, Vitek2 GN and GP cards (BioMérieux, l’Étoile, France) were used, according to manufacturers indications.

A total of 57 different milk pathogens were identified, with Staph. spp. holding the main prevalence of 27.17% (50/184, IC 95% 20.89-34.21) followed by E. coli with a prevalence of 25% (46/184, IC 95% 18.92-31.90), Proteus mirabilis with a prevalence of 9.24% (17/184, IC 95% 5.47-14.38), Enterococcus faecium with 7.61% (14/184,IC 95% 4.22-12.44), Staph. pseudintermedius with 7.07% (13/184, IC 95% 3.82-11.78), Staph. simulans with a prevalence of 5.43% (10/184, IC 95% 2.64-9.77), Agrobacterium radiobacter with 4.89% (9/184, IC 95% 2.26-9.08), Pseud. aeruginos and Staph. xylosus with 3.80% (7/184, IC 95% 1.54-7.68) of isolated strains, each followed by Staph. hominis ssp hominis with a prevalence of 3.26% (6/184, IC 95%1.21-6.96) and Staph. intermedius, M. luteus, Enterococcus faecalis and Streptococcus spp. with 5% (5/184, IC 95% 0.89-6.23) each, the remaining strains posing a prevalence under 5%.

Nosocomial strains such as Agrobacterium radiobacter, Staph. hominis ssp hominis, Burkholderia cepacia, Chryseomonas luteola, Chromobacterium violaceum,
Ochrobactrum anthropi, Staph. angiosus, Staph. capitis, Staph. cohnii cohnii, Staph. haemolyticus and Staph. warneri (MATTHAIOU et al., 2011; CHIHAB et al., 2004; MOORE et al., 2001; ELSAYED and ZHANG, 2004; HAGIYA et al., 2013; JUNCKERSTORRF et al., 2014; CUI et al., 2012; SOLDERA et al., 2013; CONE et al., 2005; DANIEL et al., 2014; IVIĆ et al., 2013) had also been isolated.

In chapter 13, "Biochemical and haematologic changes in lactating bitches", in order to evaluate haematological and biochemical changes during bitch mastitis cases, 100 lactating dams were included in a case-controlled study, but because of owners reluctance, we were only able to obtain 74 blood samples.

Dams in this study were divided into two groups. Females with primary mastitis and females with secondary mastitis. During the clinical examination, females with concurrent diseases were included in the second category (i.e. secondary mastitis), whereas clinically healthy females or just with clinical or subclinical mastitis, were included in the first category.

Haematology and biochemistry assays were performed on venous blood after a thorough local disinfection with betadine (Betadine 100 ml, Egis Pharmaceuticals Ltd., Budapest, Hungary). After collection, blood samples were rushed as quickly as possible to the laboratory at the University of Agricultural Science and Veterinary Medicine Cluj-Napoca. Complete blood count was determined with Abacus Junior Vet analyzer (Diatron Messtechnik, Budapest, Hungary) whilst differential blood count was counted on blood film stained with DiaQuick Panoptic dye kit (Reag-Fix Panoptic, Reagens Kft., Budapest Hungary). Biochemical results for total serum protein, were obtained with a Spectrophotometer analyzer UV-VIS Screen Master Touch (Hospitex Diagnostix, Fiorentino, Italy).

In secondary mastitis, one should always carefully interpret blood test and treat the dam, according to the underlying cause. In primary mastitis, haematology and biochemical assays proved to be modest and limited.

In primary mastitis bitches, Hp levels varied between undetectable (i.e. 0.000 mg/ml) and 4.333 mg/ml with an average of 1.110 mg/ml. In females with secondary mastitis, Hp levels varied between undetectable and 4.252 mg/ml with an average of 1.246 mg/ml (Table 23).

In the first category, based on each type of mammary gland diagnostic, mean Hp value was 0.453 mg/ml in females with retention mastitis, 1.274 mg/ml in congested mammary glands, 1.318 mg/ml in cases of Mastitis acuta and 1.806 mg/ml in bitches with subclinical mastitis (Table 24).

In Chapter 14, "Bitch mastitis diagnosed by milk C-reactive protein level", both local (milk) and peripheric (venous blood) CRP bitch mastitis diagnostic potential were assessed. In this chapter, a total of 45 bitches were included in 2 different groups. Group I (control group) consisted of 10 clinically healthy lactating bitches. Group II (test group) consisted of 35 bitches of 19 different breeds, with diagnosed clinical or subclinical mastitis. Serum and milk CRP were quantified using previously described canine specific TR-IFMA method (PARRA et al., 2006). Canine serum sample with known CRP concentration was used as standard. Serum samples and standards were diluted 1:7500, whereas milk samples were diluted 1:200 in assay buffer. CRP in milk samples showed a variation coefficient below 15%.
The overall diagnostic capacity of serum and milk CRP was assessed by receiver operating characteristic analysis (ROC) based on the measurements of both parameters in the 2 groups of dogs. The parameters were deemed to be efficient markers of systemic inflammation if the 95% confidence interval (CI) of the area under the ROC curve (AUC) exceeded 0.8 (CHRISTENSEN et al., 2014). The diagnostic potential of serum and milk CRP as markers of mastitis was compared by use of the AUCs obtained by ROC analysis (GARDNER and GREINER, 2006), the D'Agostino–Pearson omnibus normality test, alongside with the Friedman test and with Dunn posttest.

Serum CRP concentrations ranged from 2.0 to 8.6 μg/mL in healthy and from 0.3 to 162.3 μg/mL in diseased bitches (P=0.007). Milk CRP concentrations ranged from 0.1 to 4.9 μg/mL in healthy and from 0.3 to 40.0 μg/mL in diseased animals (P=0.002).

Statistically significantly higher serum CRP concentrations were detected in bitches suffering from clinical and subclinical mastitis than healthy dogs (P<0.05 and P<0.01, respectively). In a similar manner, statistically significantly higher milk CRP concentrations were detected in bitches suffering from clinical and subclinical mastitis in comparison with healthy dogs (P<0.05 and P<0.01, respectively). No statistical significance was detected between bitches suffering from clinical or subclinical mastitis forms.

When dogs with clinical mastitis were subdivided according to the type of pathology presented the highest serum CRP concentrations were observed in bitches suffering from gangrenous mastitis, followed by galactostasis, mammary congestion, and the lowest serum CRP concentrations were observed in cases of acute mastitis (Table 25). Milk CRP concentrations were highest in bitches suffering from Gangrenous mastitis, followed by acute mastitis, galactostasis, and the lowest in cases of mammary congestion (Table 25). However, no statistical significance was detected in serum or milk CRP concentrations when different groups of bitches with clinical mastitis were compared.

In conclusion, the present study indicates for the first time that CRP can be measured in milk of bitches. Furthermore, the results indicate the utility of CRP measurement in serum and milk in order to diagnose canine mastitis. However, it seems that milk CRP could have greater diagnostic potential compared with serum CRP, since greater area under the ROC curve for the milk CRP measurements relative to serum CRP was observed.

**GENERAL CONCLUSIONS**

1. Bitch milk studies have shown that milk cytology, pH and APPs evaluation holds great early diagnostic potential in bitch mastitis.
2. It has been shown that lactation period holds great influence regarding the type of mastitis encountered in bitches, thus, the most subclinical cases (22.05%) were encountered in the postpartum period. *Mastitis acuta* cases were in equal manner (13%) diagnosed in both antepartum and *Lactatio sine graviditate* bitches. From dams with *Lactatio sine graviditate* the majority (65.22%) were diagnosed with retention mastitis.
3. Milk cytology evaluation has proved to be efficient in the evaluation of lactating mammary glands. Henceforth, correlated with case history and clinical examination, presence of eosinophils on milk smears are conclusive for a retention mastitis diagnostic, whilst presence of numerous degenerated neutrophils alongside activated macrophages, phagocytosis and bacteria on milk specimens is consistent with an acute mammary inflammatory process.

4. Milk pH evaluation in bitch milk highlighted the presence of an alkaline reaction in most of the tested samples, from clinical and subclinical mastitis cases.

5. From *Lactation sine graviditate* bitches, 91.67% of mammary secretions had an alkaline pH value. From all cases of retention mastitis, 95.24% of obtained values were also alkaline. However, from retention mastitis *Lactatio sine graviditate* affected bitches, all milk samples tested were 100 alkaline.

6. Nosocomial potential bacterial strains presence in bitch milk should emphasize breeders, owners as well as veterinary staff about the possibility of hazardous pathogens transfer between them and lactating dams, irrespective of the type of lactation. Kennel, shelters and litter hygiene needs to be properly addressed alongside with a low manipulation of the lactating mammare as possible.

7. Bitch milk and serum CRP evaluation showed that there is a tight correlation between the two, henceforth, in order to diagnose mastitis, the local mammary value is superior to the periferic one.

**RECOMMENDATIONS**

1. In order to diagnose subclinical mastitis connected with litter disease, the early use of laboratory tests described in the present study for evaluation of the milk quality is highly recommended.

2. In order to minimize possible infections throught manual contact, regardless the lactation period, it is highly recommended to limit as much as possible mammary gland contact alongside with providing a healthy and clean environment.

3. Recalling the high incidence of retention mastitis throught *Lactatio sine graviditate* dams, in order to avoid septic mastitis, monitoring the mammary glands from this category should be made by qualified staff starting from the lutheal phase, thus avoiding a possible fatal outcome.
REFERENCES


33. TOYDEMIR T.S.F., A.F. BAĞCİGİL, N.Y. ÖZGÜR, İ. KIRŞAN, 2015, Examination of aerobic bacteria from milk samples of bitches with clinical mastitis, J Fac Vet Med Istanbul Univ, 41(2), 227-231.