Laboratory Methods Used for Early Diagnosis in Bitch Mastitis

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Bulletin UASVM Veterinary Medicine 72(1) / 2015,
Print ISSN 1843-5270; Electronic ISSN 1843-5378
DOI:10.15835/buasvmcn-vm: 11026

Abstract

The aim of this paper is to review the existing literature regarding the current methods used to diagnose bitch mastitis. Compared to the information available on ruminant mammary gland inflammation, the data regarding bitch mastitis is limited.

So far microbiology assays are most widely used to identify, (based on antibiograms) and to treat mammary gland infections. Milk cytology has also been taken into consideration as a diagnostic tool. Clinicians have also started to use haematology, biochemistry and pH assays to confirm clinical signs of mastitis.

It is well known that one of the causes of neonatal mortality is undiagnosed episodes of mastitis. Early diagnosing of subclinical cases of glandular tissue inflammation and avoiding chronic episodes of infection, can limit neonatal mortality.

Keywords: *bitch, cytology, mastitis, microbiology, milk.*

INTRODUCTION

Mastitis represents the inflammation of the mammary gland tissue (Tudor, 2004; Boden, 2005). Usually, mastitis is a disease of bacterial etiology (Orfanou *et al.*, 2008), but parasitical etiology has also been reported (Park *et al.* 2007). In human medicine there are reports of sarcoidosis, histoplasmosis and aspergillosis mastitis episodes (Gansler and Wheeler, 1984; Houn and Granger, 1991; Govindarajan *et al.* 1993; Pinto and Mandreker, 1996).

In most cases, the infection appears in the postpartum period, but it can also occur in the lactating nursing bitch, even in *Lactacio sine graviditate* scenarios (Merk Veterinary Manual, 2010). Mastitis can affect one or more sections of the glandular tissue or one or more mammary glands. Mammary infections can be acute and life threatening (Johnston *et al.*, 2001). The

females display general symptoms (i.e. vomiting, dehydration, anorexia, fever, trembling, decreased mothering) as well as local signs of illness (i.e. discolored milk, erythema, oedema, hardening and pain of the affected glandular tissues). There are also reported cases where the dams show no signs of illness, but the offsprings failed to thrive (Johnston *et al.*, 2001; Wiebe and Howed, 2009; Merk Veterinary Manual, 2010; Lopate, 2012, Casal 2014 *in press*).

When dealing with mammary gland infections, the diagnostic is usually limited to clinical examination. Milk microbiology (Jung *et al.*, 2002), cytology (Olson and Olson, 1984) and pH test (Hasegawa *et al.*, 1993) have also been used, not only to confirm clinical episodes of mastitis, but also to identify subclinical or chronic cases.

There are few reports in the literature where hematology and biochemistry assays have

been taken into consideration when diagnosing mammary gland infections (Hasegawa *et al.*, 1993; Ververidis *et al.*, 2007).

Given the fact that bitch mastitis is commonly overlooked, early diagnosis of subclinical mastitis episodes by establishing a clear pathway to identify mammary gland inflammation, can significantly help to reduce neonatal mortality.

The aim of this review is to show practitioners worldwide the necessity of correctly diagnosing mammary gland infections in dams, whereas indiscriminate use of antibiotics may create resistant bacterial strains, in both bitch and pups.

Milk microbiology

For microbiology assays, milk is usually collected by manual expression (first milk drop being discarded) into sterile vials (Martín *et al.*, 2010), sterile swabs with transport medium (Milani *et al.*, 2012) or even directly on to sterilised bacteriological loops (Ververidis *et al.*, 2007). Even the fine needle aspirate technique has been used (Sangha *et al.*, 2011).

Standard microbiology techniques for isolation and identification of bacteria are currently used. A loop full of mammary secretion is plated onto blood agar, (especially for isolation of *Staphylococcus* and *Streptococcus* spp.) and McConkey (selective for some *Enterobacteriaceae*), being incubated for 24-72 h at 37°C in aerobic and modified atmosphere (5% CO₂) conditions (Schäfer-Somi, 2003). Initial identification of bacteria is performed by macroscopic observation of colonies, Gram stain, cellular morphology, oxidase, catalase, mobility and oxidation-fermentation tests, type of haemolysis for *Streptococcus* spp., and coagulase for *Staphylococcus* spp. (Milani *et al.*, 2012). Biochemical incorporated API systems commercial tests are used to identify bacterial species (Schäfer-Somi, 2003). Additionally, after two days enrichment in Thioglycollate medium fluid and Trypticase Soy broth, cultural procedures on solid media are performed (Schäfer-Somi, 2003).

For lactobacilli isolation, milk samples are diluted in peptone water, then plated onto Man. Rogosa and Sharpe agar plates and then aerobically incubated at 37°C for 24 h. The same samples are cultured on MRS supplemented with L-Cysteine (0.5g/L), (MRS-Cys) agar plates, anaerobically (85% nitrogen, 10% hydrogen, 5% carbon dioxide) at 37°C for 48 h (Martín *et al.*, 2010).

Staphylococcus pseudintermedius and *Streptococcus canis* are very important pathogens in dogs and cats (Weese, 2008). Results of microbiology assays show a relatively wide spectrum of bacteria isolates. So far, from bitches with *mastitis acuta*, haemolytic *Staphylococcus* spp., *Staph. intermedius*, *Staph. haemolyticus*, β haem. *Streptococcus*, *Klebsiella pneumoniae*, *E. coli* and *Proteus mirabilis* have been identified (Schäfer-Somi, 2003).

From asymptomatic females, *Staph. intermedius*, haem. *Staphylococcus* spp., *Staph. epidermitis*, *Staph. simulans*, β haem. *Streptococcus*, *E. coli*, *Enterococcus durans*, *P. stutzeri*, *Shigella* spp., *Acinetobacter anitratus* and *Bacillus* spp. have been isolated (Schäfer-Somi, 2003). Jung *et al.* (2002) also isolated α -hemolytic *Streptococci* and γ -haemolytic *Streptococci*.

Araújo *et al.*, (2011) proved that *Staph. hyicus* can produce mammary gland inflammation accompanied by lymphadenitis, by isolating the pathogen from a three-year-old Pitt bull female, which died after *amastitis acuta* episode (Araújo *et al.*, 2011). There is also one experimental bitch mammary gland infection with a *Staph. intermedius* strain in six primiparous Beagle-breed females (Ververidis *et al.*, 2007).

Martín *et al.*, (2010) managed to isolate lactobacilli (*L. murinus*, *L. animalis*, *L. reuteri*, *L. johnsonii* and *L. fermentum*) and *E. faecium*, *E. faecalis*, *Strept. salivarius*, *Staph. epidermidis*, *Strept. bovis*, *Staph. simulans*, *Staph. pseudintermedius*, *W. viridescens* and yeast from bitch milk.

However, enterococci can be opportunistic pathogens and may cause a range of different infections in animals and humans, including mastitis. *E. faecalis* and *E. faecium* strains isolates, constitute a pathogenic reservoir for offsprings (Jiménez *et al.*, 2013).

It has also been suggested that bacteria isolated from the mammary gland and vagina, are responsible for neonatal infections (Sager and Remmers, 1990; Münich and Lübke, 2004). In order to eliminate bacterial flora and hopefully, to eliminate the neonatal mortality risk, many dog breeders treat dams before and after parturition with antibiotics. Unfortunately, this practice will only predispose the vagina to colonisation by opportunistic pathogens and will select resistant bacteria strains (De Graef *et al.*, 2004; Rota *et al.* 2011).

Because milk is acidic, weak base antibiotics distributes and gets trapped into it. Macrolids, amoxicillin-calvulanated or cephalexin may be empirically initiated until culture results return (Martí, 2009).

Milk biophysical parameters

Due to the limited amount of milk acquired from dams, milk pH values are recorded by using litmus paper (Dziecioł *et al.*, 2006). Parameters such as chloride levels have also been measured (Hasegawa *et al.*, 1993; Dziecioł *et al.*, 2006).

Milk pH values obtained so far (Hasegawa *et al.*, 1993, Dziecioł *et al.*, 2006) suggests that females suffering from *mastitis acuta* have an alkaline pH value of 7, whilst females diagnosed with *Lactatio sine graviditate* have a more basic pH value (i.e. 6.7). Tests have shown that healthy dams have a more acid pH level of about 6.3 averages.

Chloride levels registered show an increase in (mean=199mg %) cases of mastitis. Healthy females and females with *Lactatio sine graviditate* have been found to have similar results, 84.3mg %, respectively 84.1mg% (Dziecioł *et al.*, 2006).

Milk cytology

Milk cytology is carried out by using secretion films made by the direct smearing squash technique (Meyer *et al.*, 2010) using about 20 µl of each collected sample on microscope objective plate.

The squash technique is an adaptable procedure for management of semisolid, mucus-like or pelleted by centrifugation cytology specimens. A small amount of material is placed on a clean glass slide, approximately 1 cm (1/2 inch) from the frosted end. A second clean slide is placed over the specimen at right angles. The specimen is gently but firmly compressed between the two glass slides and in the same continuous motion the top slide is pulled along the surface of the bottom slide, directing the material away from the frosted end. The objective is to redistribute the material, turning a multicellular mass into a thin monolayer ideal for maximal flattening of individual cells and even stain penetration. The compression preparation thus optimizes the specimen for microscopic examination of cell morphology. A properly prepared glass slide is characterized by a feather-shaped area with a monolayer end referred to as the "sweet spot", (Sabău and Rotaru, 2006a; Meyer *et al.*, 2010).

Smears are stained by using the Giemsa (Ververidis *et al.*, 2007), Wright-Giemsa (Olson and Olson, 1984) or Wright-Leishman (Sangha *et al.*, 2011) technique. Romanowsky-type stains are preferred because they work rapidly and are easy to use (Sabău and Rotaru, 2006a; Meyer *et al.*, 2010). For diagnostic, in ruminant mastitis, the May-GrunwaldGiemsa stain technique is the most used amongst the Romanowsky-type stains (Sabău and Rotaru, 2006b).

The percentage of leukocyte subpopulation is determined by distinguishing types of inflammatory cells within the films (Ververidis *et al.*, 2007). For total cell counts, Olson and Olson, (1984) use a white blood cell (Unopette – Becton-Dickinson) and hemacytometer counting chamber, whereas 20 µl of milk is placed in Unopette diluent. To aid differentiating inflammatory milk cells from fat droplets, 20 µl of Diff-Quick solution II is added. Total cell counts of milk samples are expressed as cells/µl of milk (Olson and Olson, 1984). The Whiteside test has also been used to confirm clinical mastitis (Ververidis *et al.* 2007).

Few studies have looked into bitch milk cytology. There is one study carried out in 1984 that evaluates canine milk cytology (Olson and Olson, 1984). This study shows cytology variations among glands from the same dam. In canine milk, macrophages and polymorphonuclear leukocytes are common isolates. Macrophages with ingested milk fat vary greatly in size. Even in milk of healthy bitches, polymorphonuclear leukocytes present pyknotic nuclei. Severely degenerated neutrophils with engulfed bacteria are seen in milk collected from females that suffer from septic mastitis (Olson and Olson, 1984).

Plasma cells, lymphocytes, cells with degenerated nuclei and round nuclei are also present. In milk samples collected from bitches resorbing milk, fewer fat droplets are present than in samples collected from milking females (Olson and Olson, 1984). Bacteria from healthy dams, resulting from skin contaminants are present in milk samples, unless bacteria are accompanied by increased cell counts and phagocytosis, proof of mammary gland infection (Olson and Olson, 1984). Galactostasis cases are accompanied by an increased count of eosinophils. Engulfed blood pigment or erythrocytes are evidence of milk blood traces (Olson, 1984).

Milk inflammatory cell response (macrophagic foamy cells, neutrophils and eosinophils) have been also reported in a 6-year-old Bernese female dog living in Italy, presenting with a breast lump. Cytology examination of the nipple secretion fluid smears showed the presence of glandular cell clusters and of some nematodes (*Dirofilaria repens*) of about 350 µm in length in the background (Manuali *et al.*, 2004).

There are reports in human medicine of breast inflammatory lesions with various causes, including actinomycosis in acute, subacute mastitis and abscess. As a response to the injury, numerous polymorphonuclear leukocytes, scattered ductal cells and necrotic material are found on smears (Koss and Melamed, 2006). In chronic cases, especially in developing countries, breast tuberculosis lesions, such as columnar, oddly shaped and multinucleated giant cells surrounded by inflammatory infiltrate, have been reported (Shinde *et al.*, 1995).

Some authors (Fontaine *et al.*, 2007) suggest that bitch milk cytology has no clinical importance. Sangha *et al.* (2011) proved that milk cell counts increase during bitch mastitis episodes by comparing cytological findings with histological diagnose. A 70% concordance had been found by correlating these assays (Shanga *et al.*, 2011).

Dziecioł *et al.* (2006), during their research, found an increased somatic cell count in bitch milk. Two weeks from delivery, mean somatic cell count were $< 3 \times 10^6 \text{ ml}^{-1}$. Between 3-5 weeks after delivery, mean values were about $< 3,8 \times 10^6 \text{ ml}^{-1}$ and 6-8 weeks after delivery mean values were about $< 4,9 \times 10^6 \text{ ml}^{-1}$ (Dziecioł *et al.*, 2006).

In vivo experiments (Ververidis *et al.*, 2007) showed an increased somatic cell count at 12 h after inoculation of *Staph. intermedius* in the right caudal abdominal mammary gland of six Beagle females, and remained high through lactation. In samples taken from control glands, the values also increased 12 h after inoculation but did not remain high during lactation (Ververidis *et al.*, 2007).

In the examined secretion films, neutrophils predominate (>75%) in initial stages. During the 11th day post challenge, the proportion began to reduce. From the 3rd day post challenge, degenerated neutrophils were already evident. Lymphocytes were also present (20%) after the 6th day of challenge and began to predominate from day 14. Similar findings were seen in the control

glands. However, macrophages and lymphocytes were found in a high proportion (>25%), but in fewer numbers in later stages (Ververidis *et al.*, 2007).

Cytology should be interpreted with caution as it is normal for low numbers of neutrophils and eosinophils to be present in healthy bitch milk. However, large numbers of neutrophils and presence of engulfed bacteria is consistent with infections (Olson and Olson, 1984). Keeping in mind the indiscriminate use of antibiotics, treatment should at least be based first on cytological examination of milk secretion (Lopate, 2012).

Blood test

For hematology and biochemical assays, blood is harvested from the jugular vein into heparin- or EDTA-containing vials. Standard full hematological tests are carried out by using blood-analyzer. Albumin, total serum proteins, globulins (Hasegawa *et al.*, 1993; Ververidis *et al.*, 2007) and haptoglobin measurement (Dziecioł *et al.*, 2006), are carried out using biochemical analyzers (Ververidis *et al.*, 2007).

To the author's knowledge, there is only one documented case (Hasegawa *et al.*, 1992) of *mastitis gangrenosa* in bitch, where biochemical and hematological changes had been recorded (Hasegawa *et al.*, 1992).

Laboratory findings showed marked leukocytosis (36,300/µl), marked increase of α, β and γ-globulins, mild anemia, slight increase of plasma alkaline phosphatase and creatine phosphokinase levels, mild hypernatremia and hypokalemia on the day of admission. Additionally, large platelets were observed in the blood smears. The number of circulating leukocytes, temporarily decreased (26,000/µl) on day 1. Increased peripheral leukocytes (48,600/µl), decreased circulating platelets (179,000/µl), abnormal pattern of platelets and bad physical condition were recorded on the same day (Hasegawa *et al.*, 1992).

Experimental infection (Ververidis *et al.*, 2007) shows a significant overall increase of leukocyte counts after challenge. Nevertheless, after 12 h, there was a decrease of leukocyte counts, followed by a sharp increase. Leukocyte counts had returned to pre-challenge values after the 6th day. After challenge, the neutrophil counts had significantly increased. Total neutrophil

count returned to pre-challenge values after day 6. Twelve hours after challenge, there was a significant platelet count decrease, followed by an increase during the fourth day.

There were no significant changes in the globulin, albumin and haematocrit values during this experiment (Ververidis *et al.*, 2007).

Some author's (Lopate, 2012) signaled that in cases of mastitis, leukocytosis, with or without a degenerative left shift, eosinophilia, dehydration, increased renal and hepatic enzymes and electrolyte abnormalities may be found (Lopate, 2012).

Acute-phase proteins

In recent years, in veterinary medicine, Acute-phase proteins (APPs), such as Haptoglobin (Hp), serum amyloid A (SAA), C-reactive protein (CRP), α_1 -acid glycoprotein (AGP), fibrinogen (Fb) and albumin (Eckersall, 1995) had started to be used to diagnose and assess inflammatory processes (Ceron *et al.*, 2005). These proteins are also used to evaluate the evolution of selected diseases (Kocaturk *et al.*, 2010). This review aims to highlight the use of Haptoglobin as a potential marker for bitch mammary gland infections, by comparing the available data for bitch and cow mastitis.

The acute phase response is stimulated by the release of cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor α (TNF α). Inflammatory lesions or infections stimulate macrophages and monocytes to release this cytokines (Eckersall, 2000).

The acute phase proteins are synthesized by hepatocytes upon stimulation of the pro-inflammatory cytokines, released into the blood stream (Gruys *et al.*, 1994; Eckersall, 2000; Ceron *et al.*, 2005).

In dogs, APPs are used to diagnose and assess the health status of individuals suffering from various diseases (Eckersall, 2000; McGrotty *et al.*, 2003; Maier *et al.*, 2004; Ulutaş *et al.*, 2007). Lately, these proteins are used to assess the health status of breeding bitches as well. Changes in APPs (serum amyloid A, C-reactive protein, haptoglobin, ceruloplasmin and fibrinogen) produced in response to ovariohysterectomy in healthy bitches and those with pyometra, including APPs levels in pregnancy and oestrus cycles have been assessed (Dąbrowski *et al.*, 2007; Ulutaş *et al.*, 2009; Serin and Ulutaş, 2010; Dąbrowski *et al.*, 2013).

Dziecioł *et al.*, (2006) focused their research on Hp levels in lactating bitches. The level of Hp indicating inflammation ranged between 4-9 mg/ml (Dziecioł *et al.*, 2006). Haptoglobin increase is strictly correlated with the degree of tissue damage. This particular APP reaches the maximum concentration, after tissue damage in 3-5 days (Kostro *et al.*, 1996; Cerón *et al.*, 2005).

Haptoglobin concentration reflects the intensity of inflammatory processes irrespective of the site of blood collection (Dąbrowski *et al.*, 2013). C-reactive protein and serum amyloid A react rapidly, whilst Hp reacts slowly (Dąbrowski *et al.*, 2009). Haptoglobin in dogs is considered to be a moderate APP, as it increases 2-3-fold in response to infection, trauma or inflammation (Eckersall 1995; Eckersall and Conner, 1998).

In dogs, normal Hp plasma levels range between 0-1.69 mg/L. In dogs diagnosed with polyarthritis mean Hp level registered were 11.6 mg/ml, with a range of 7.2-16.5 mg/ml (Eckersall *et al.*, 1999).

In females suffering from *mastitis acuta*, the Hp recorded levels ranged between 5.3-7.5 mg/ml. In *mastitis cronica*, the recorded levels ranged between 2.2 and 2.5 mg/ml. In bitches diagnosed with *lactatio sine graviditate*, the obtained value did not exceed 0.6 mg/ml (i.e. 9 females), (Dziecioł *et al.*, 2006). Usually, in dogs, 24 h after surgical trauma, the Hp level increases 2-5 fold (Cerón *et al.*, 2005).

In ruminants, Hp is a valuable marker of disease. It is considered as a major APP (Eckersall *et al.*, 1999; Eckersall, 2000). In cows, the normal Hp level ranges between 22-47 mg/L. Experimental *E. coli* mastitis infection in cows, showed a significant Hp level increase (i.e. 52-fold). Furthermore, 12 hours after challenge, the haptoglobin value already started to increase, whilst the highest Hp levels were recorded between 48-120 hours after challenge (Salonen *et al.*, 1996).

Other experimental infections in cows, recorded no Hp value before bacterial challenge. The maximum obtained value was reached 2-3 days post-challenge. Haptoglobin levels have predictive accuracy as well (Hirvonen *et al.*, 1996). However, serum Hp does not always correlate with severity of disease and could not discriminate the fatal *E. coli* cases (Hirvonen *et al.*, 1999).

Furthermore, rapid increase of milk somatic cells after repeated challenges indicates that milk

inflammatory cells are primed by previous *E. coli* challenge. Subsequent Hp challenges do not increase at the same level as in the first challenge scenarios (Salonen *et al.*, 1996).

In subclinical infections, the pro-inflammatory cytokines increase. Henceforth, APPs levels are used in slaughterhouses as indicators of food safety. Mean Hp herd levels are also related to the hygiene level of the production facilities (Eurell *et al.*, 1992; Kostro *et al.*, 1996; Petersen *et al.*, 2003). Haptoglobin level is also used in swine husbandry, as a biomarker for weight gain (Eurell *et al.*, 1992).

CONCLUSIONS

Because bitch mammary infections are widely overlooked, extra care should be taken into evaluating post-partum pathology. Bacterial pathogens that cause mammary infections can migrate from the uterus and vagina to the mammary glands (haematogenous transmission), (Fontaine *et al.*, 2007).

Graham and Taylor (2012) suggest that exogenous infections of the mammary glands are rare and commonly infections of the reproductive tract are of endogenous origin. Many of the bacterial pathogens involved in the reproductive tract are part of the urogenital microflora. Therefore bacterial infections (i.e. bacteremia and endotoxemia) are opportunistic and predisposing factors must be present for the disease to develop, thus establishing a definitive diagnosis can be challenging (Graham and Taylor, 2012).

Bacteria strains from skin and oral mucosa of both, the dam and the offspring, can be responsible for mammary gland infections (exogenous transmission), especially in *Lactatio sine graviditate* scenarios, where the bitch frequently licks the mammary glands (Jung *et al.*, 2002).

There are also reports were the same bacterial strains had been isolated from bitch milk and puppies diagnosed with septicaemia (Münnich and Lübke, 2004; Rota *et al.*, 2011). Conditions such as pyometra, metritis or puerperal vaginitis can predispose the nursing dams to glandular tissue inflammation, predisposing the litter to septicaemia, neonatal mortality or “fading puppy syndrome” (Schäfer-Somi *et al.*, 2003, Milani *et al.*, 2012).

Endotoxemia is also a potentially fatal sequel to mastitis (Graham and Taylor, 2012). Reports of neonatal septicemia caused by a *Streptococcus*

dysgalactiae subs. *dysgalactiae* strain isolated from two dead puppies have also been related (Vela *et al.*, 2006).

Mastitis gangrenosa has also been signaled in cats (Wilson, 2013). Furthermore, there are reports in the literature where fibroadenomatous hyperplasia in cats complicate with mammary abscess (Burstyn, 2010) highlighting the important role of mammary gland infection management.

Milk samples should be obtained for cytology and microbial culture and susceptibility tested before antibiotic therapy is initiated (Casal, 2014, *in press*). Early diagnosis of a subclinical mammary infection can help prevent postpartum complications (i.e. mortality in pups, antibiotic resistance). Avoiding prophylactic administration of antibiotics is one of the major advantages of early diagnosing mastitis episodes.

In mammary cancer, the prognosis is guarded to poor (Rutteman and Kirpensteijn, 2003), whilst in mastitis, except for gangrenous cases, the prognosis is usually good. Given the circumstances, it is of the uttermost importance to differentiate between a mammary inflammatory response and mammary neoplasia, both in human and veterinary medicine. By correctly interpreting mammary or milk cytology, a correct diagnosis and differentiation can be made between the two mentioned morbid entities.

Because dogs and people live in close contact, risk of bacteria transfer from one to the other is high (Wedley *et al.*, 2011). Transfer of milk pathogens from humans to bitch milk is therefore possible. Extra care should be provided when handling puppies and the bitch's mammary glands by owners and veterinary staff.

Laboratory test (i.e. pH, cytology, biochemistry, hematology, microbiology) should become the golden standard when veterinary specialists start to manage mammary gland infections. In cows, when milk samples are not available, Hp is used as a mastitis indicator (Eckersall *et al.*, 1999). In order to early diagnose a bitch mammary gland infection, further research is required to precisely establish which parameters better suits for a clear diagnostic.

Acknowledgements. The warmest of thanks to TressaRebek-Nagy for helping with English grammar and spelling.

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