Fibrinogen and Ceruloplasmin in Plasma and Milk from Dairy Cows with Subclinical and Clinical Mastitis

1A. Davasaz Tabrizi, 1R.A. Batavani, 1S. Asri Rezaei and 2M. Ahmadi
1Department of Clinical Science, 2Department of Microbiology,
Faculty of Veterinary Medicine, University of Urmia, P.O. Box 1177, Urmia, Iran

Abstract: The potential using of Acute Phase Proteins (APPs) in the assessment of mammary gland health was studied by examining the levels of Fibrinogen (Fb) and Ceruloplasmin (Cp) in plasma and milk from dairy cows with different grades of mastitis. Plasma samples were taken from jugular vein and milk samples were collected from quarters of cows with subclinical and clinical mastitis, as well as healthy controls. California Mastitis Test (CMT) were performed on each udder quarter of cows for detection of CMT2+ and CMT3+ quarters. CMT (0) and culture negative cases were considered healthy cows. Clinical mastitis, was graded as mild (clots in milk) or moderate (clots in milk and visible signs of inflammation in the mammary gland(s)). The concentrations of Fb in the plasma of the cows with subclinical and clinical mastitis were higher than in the plasma of the healthy cows (p<0.01). There was no significant difference in plasma concentration of Cp between healthy and subclinical groups (p>0.05), but differences between clinical and healthy groups were significant (p<0.05). The concentrations of Fb and Cp in the milk of the cows with subclinical and clinical mastitis were higher than in the milk of the healthy cows (p<0.01). The results indicated that measurement of Fb in plasma and milk and Cp only in milk might be suitable for early diagnosis of mastitis in dairy cows.

Key words: Acute phase proteins, udder inflammation, blood, milk, cow

INTRODUCTION

Mastitis is the most frequent and expensive disease associated with current intensive dairying (Whitaker et al., 2004). It has adverse effects on the economics of milk production by reducing the quantity and quality of milk (De Graves and Fetrow, 1993) and increasing expenses through the imposition of financial penalties by milk purchasers for high numbers of somatic cells in milk as a result of mammary infections (Booth, 1997). Per acute clinical mastitis has also been recognized as the major cause of mortality in adult dairy cows (Menzies et al., 1995). Mastitis is caused by several species of common bacteria, fungi, mycoplasma and algae. Subclinical infections are those for which no visible changes occur in the appearance of milk or the udder, but milk production decreases, somatic cell count increases, pathogens are present in the secretion and composition is altered. Clinical mastitis is recognized by abnormal milk, varying degrees of mammary gland inflammation (redness, heat, swelling, pain) and with presence or absence of illness in the cow. Milk production declines, bacteria are present in the milk and the milk can vary from having a few milk clots to serum with clumps of fibrin in the secretion (Tyler and Cullor, 2002). Early identification of udder health problems is essential for dairy farmers and veterinarians to ensure not only animal well-being but also milk quality and dairying productivity. Economic aspects interfere with the routine application of bacteriologic examination of quarter milk samples. For this reason, alternative parameters are used to identify trends in the development of the udder health in a dairy herd, although these parameters indicate inflammation. Acute Phase Proteins (APPs) are a group of serum proteins which undergo substantial changes in concentration following infection, inflammation or trauma (Gruys et al., 1994). Fb is involved in homeostasis, providing a substrate for fibrin formation and in tissue repair, providing a matrix for the migration of inflammatory related cells (Thomas, 2000).

Fb is used in cattle as a reliable indicator of the presence of inflammation, bacterial infection or surgical trauma (Hirvonen et al., 1996; Cheryk et al., 1998; Hirvonen and Pyyromaa, 1998). Cp is a copper-containing ferroxidase that oxidizes toxic ferrous iron to its nontoxic ferric form (Patel et al., 2002). It protects tissues from iron-mediated free radical injury and is involved in various antioxidant and cytoprotective activities (Inoue et al.,

Corresponding Author: Afshin Davasaz Tabrizi, Department of Clinical Science, Faculty of Veterinary Medicine, University of Urmia, P.O. Box 1177, Urmia, Iran  Tel: +98-441-2774737  Fax: +98-441-2777099
1999). The application of Cp to diagnosis remains less common than that of other APPs, but there have been a number of studies confirming that this ferroxidase is an indicator of infection in cattle (Chassagne et al., 1998; Sheldon et al., 2001).

The aim of this study was to evaluate the potential of using APPs in the assessment of mammary gland health by examining the levels of Fb and Cp in relation to different grades of subclinical and clinical mastitis in dairy cows.

MATERIALS AND METHODS

Animals were selected during the year 2006 from two Holstein dairy herds located around the city of Tabriz in East Azerbaijan province of Iran. Cows were milked three times daily by machine milking. All cows were subjected to post-milking teat disinfection, those were dried off approximately 2 months before expected calving and all quarters of cows were infused with an antibiotic preparation approved for use in non-lactating cows following the last milking of lactation. Milk samples were collected from cows just before morning milking. Teats were washed thoroughly and dried with a single-use paper towel. The first three streams of milk from each teat were discarded. The teat end and orifice was sanitized with cotton swabs soaked in 10% ethyl alcohol and approximately 10 mL foremilk sample were collected from each quarter of cow in a sterile tube held horizontally. Clinical mastitis was recognized by the dairymen on each farm in the usual way, by observation and palpation of the udder. In 25 of the cases, mastitis was diagnosed by the presence of clots in the milk (defined as mild mastitis) and in 25 other cases by the presence of clots and observable inflammation in the infected quarter such as heat, pain, redness or swelling (defined as moderate mastitis). Subclinical mastitis was determined by CMT. The CMT results were interpreted as negative (C), 1+ (traces), 2+(gel) and 3+(clumps), (Busato et al., 2000). CMT1+ cases were eliminated from this study. CMT2+ and MT3+ cases (25 case from each score) were submitted in the research. CMT0 and culture negative cases were considered healthy (Control). Milk samples were also taken from an unaffected, non-mastitic, diagonally opposed quarter of the udder of the healthy and mastitic cows, as intra-animal controls. The number of samples in each group were 25 cases. Jugular blood samples were taken from each dairy cow. Venoject tubes with EDTA and without additive were used. All milk and blood samples were tested at mid lactation and none of the cows were sampled twice in the study. Samples were immediately placed in crushed ice and submitted to the laboratory within 2-4 h. Somatic cell count were determined by a fluoro-opto-electronic method (Fossomatic 250®, Foss electric, Hillerød, Denmark). For bacteriological examinations, standard procedures were performed according to the guidelines described by Sears and McCarthy (2003) and Quinn et al. (1994). Milk serum (whey) was prepared at a two-step centrifugation procedure. At first milk samples were centrifuged at 3000 rpm for 10 min to remove their creams and cells. Samples were then treated with 0.1 M hydrochloric acid at controlled pH for 20 min for casein precipitation. Treated samples were re-centrifuged and the supernatants (Whey) were collected. Fb in plasma and milk was determined using the heat precipitation technique (Millar et al., 1971). Cp in plasma and milk was determined by its P-phenylenediamine oxidase activity (Sunderman and Nomoto, 1970).

Data were analyzed by using the Minitab statistical programme, version 14.0. One-way ANOVA was carried out to find out the differences between the results of mastitic and non-mastitic plasma and milk. Student's t-test with p<0.01 was used to evaluate differences between mastitis-healthy and mastitis quarters on dairy cows.

RESULTS

Bacteria isolated from the mastitic cows included the usual range of pathogens. The isolates from subclinical mastitis cases were coagulase negative staphylococci (40%), Staphylococcus aureus and Corynebacterium bovis (each 12%), Streptococcus sp. (6%), Serratia marcescens, Enterobacter aerogenes and Proteus sp. (each 4%). Eighteen percent from subclinical mastitis cases showed no growth on bacteriological examination. Clinical mastitis cases had the following bacteria isolated from them: Staphylococcus aureus (26%), coagulase negative staphylococci (18%), Streptococcus sp. (10%), Corynebacterium bovis and E. coli (each 8%), Pasteurella multocida (6%), Bacillus cereus and Arcanobacterium pyogenes (each 4%). Sixteen percent from clinical mastitis cases showed no growth on bacteriological examination. The somatic cell count in milk from quarters with mastitis and clinical mastitis were significantly (p<0.01) greater than in the milk of the control cows, also was significant difference between each four mastitic groups (p<0.01) (Table 1).

The mean plasma Fb in subclinical and clinical groups were significantly different with healthy groups (p<0.01). While between affected cows, Moderate mastitis group only had difference with other groups (p<0.01). The mean plasma Cp in the control group had no difference with
Table 1: Means and standard deviation of somatic cell count (SCC), Plasma Fb and CP concentrations values in different groups (n= 25 in each group).  

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Subclinical CMT2+</th>
<th>Subclinical CMT3+</th>
<th>Clinical Mild</th>
<th>Clinical Moderate</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC (1000/mL^-1)</td>
<td>11.60±2.9*</td>
<td>2800.00±1231.5*</td>
<td>6279.00±1329.5*</td>
<td>9347.00±2177*</td>
<td>12766.00±2721.5*</td>
</tr>
<tr>
<td>Plasma Fb (mg/dL^-1)</td>
<td>405.97±107.99*</td>
<td>603.40±131.9*</td>
<td>606.80±126.3*</td>
<td>618.3±120.5*</td>
<td>726.80±147.9*</td>
</tr>
<tr>
<td>Plasma CP (mg/dL^-1)</td>
<td>22.08±4.5*</td>
<td>31.2±8.7*</td>
<td>30.8±6.0*</td>
<td>36.5±6.3*</td>
<td>37.02±10.02*</td>
</tr>
</tbody>
</table>

The mean different letters (A, B, C, D, E) in each row are significantly different (p<0.01). The mean different letters (a, b) in each row are significantly different (p<0.05).

Table 2: Milk Fb mean and standard deviations values in different groups and contra lateral quarters in each group.  

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mastitic quarter (mg/dL^-1)</th>
<th>Contra lateral quarter (mg/dL^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.3±5.5*</td>
<td>9.8±4.6</td>
</tr>
<tr>
<td>CMT2+</td>
<td>27.6±8.7*</td>
<td>14.1±6.7*</td>
</tr>
<tr>
<td>CMT3+</td>
<td>32.9±12.2*</td>
<td>13.6±2.9*</td>
</tr>
<tr>
<td>Mild clinical</td>
<td>31.5±15.4*</td>
<td>14.3±3.8*</td>
</tr>
<tr>
<td>Moderate clinical</td>
<td>38.9±13.7*</td>
<td>13.0±5.7*</td>
</tr>
</tbody>
</table>

The mean with different letters (a, b and c) in the same column are significantly different (p<0.01). Common letter(s) in column explain no significant difference (p>0.05). *: p<0.01 within the row.

Table 3: Milk CP mean and standard deviations values in different groups and contra lateral quarters in each group.  

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mastitic quarter (mg/dL^-1)</th>
<th>Contra lateral quarter (mg/dL^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.9±1.02*</td>
<td>2.68±1.53</td>
</tr>
<tr>
<td>CMT2+</td>
<td>8.02±4.52*</td>
<td>4.07±2.17*</td>
</tr>
<tr>
<td>CMT3+</td>
<td>8.58±2.74*</td>
<td>3.88±2.05*</td>
</tr>
<tr>
<td>Mild clinical</td>
<td>8.64±4.87*</td>
<td>4.01±2.58*</td>
</tr>
<tr>
<td>Moderate clinical</td>
<td>11.50±3.68*</td>
<td>7.9±3.51*</td>
</tr>
</tbody>
</table>

The mean with different letters (a, b and c) in the same column are significantly different (p<0.01). Common letter(s) in column explain no significant difference (p>0.05). *: p<0.01 within the row.

Subclinical groups (p<0.05), but differences between control group and clinical groups were significant (p<0.05). However, there was no difference between mild and moderate groups (p>0.05) (Table 1).

The concentrations of Fb and CP were significantly higher in the milk of inflected quarters than those in normal milk (p<0.01). Also, difference between samples of milk from quarters with CMT2+, CMT3+ and mild mastitis groups were no significant (p>0.05), but difference between CMT2+ group and clinical moderate mastitis group was significant (p<0.01). In each mastitic group concentrations of each two APPs was significantly greater in the infected quarter than in the diagonally opposite quarter (p<0.01). On the other hand, there was no difference between healthy quarter of control group and contra lateral quarter (Table 2, 3).

**DISCUSSION**

The bacteriological results from the subclinical cases demonstrated that Coagulase Negative Staphylococci (CNS) were the most frequently isolated bacteria. CNS organisms, group of staphylococcal species, have become the predominant pathogens causing bovine mastitis in many countries (Pritikala et al., 2004; Rajala-Schulz et al., 2004). Although the CNS usually cause only subclinical or mild clinical mastitis (Horkanen-Buzalski et al., 1994), they are harmful since they increase Somatic Cell Count (SCC) in milk (Chaffer et al., 1999) and may slightly decrease milk production (Timms and Schultz, 1987). Mastitis caused by CNS responds well to antimicrobial treatment (McDougall, 1998; Waage et al., 2000; Taponen et al., 2003). Staphylococcus aureus was the second most causative organism of subclinical mastitic cows. Staphylococcus aureus is still regarded as one of the most infectious agents which produces mastitis in cattle. The other bacteria frequently was isolated Corynebacterium bovis. C. bovis are generally considered as opportunistic pathogens and inhabitants of teat canals (Rainard, 1987). The infection causes minor histopathological alterations in the udder parenchyma without affecting the secretory function of the tissue (Sordillo et al., 1989). Environmental streptococci from 5% of subclinical and 10% of clinical mastitis were isolated. In countries where the prevalence of intramammary infections due to the contagious pathogens has been reduced or eradicated, the proportion of intramammary infections associated with environmental streptococci has increased markedly (Radosits et al., 2007). The bacteriological results from the clinical cases of mastitis shows the Staphylococcus aureus is the most frequent organism. S. aureus is ubiquitous in the environment of dairy cattle. The infected mammary gland of lactating cows is the major reservoir and source of the organism. Transmission between cows occurs at the time of milking by contaminated milker's hands and teat cup liners (Radosits et al., 2007). The mean SCC was greater in the cows with moderate mastitis than those with mild mastitis, so SCC of subclinical and clinical groups were significantly higher than the SCC of the normal cows. Cell counts are used routinely for the diagnosis of subclinical mastitis but are not used for diagnosis of clinical mastitis because the visible alterations in the milk which usually accompany the increase in cells and clots and flake in the milk, make automated counting difficult. Inflammation of the mammary gland leads to a variety of compositional changes in milk either because of local effects or because of serum components entering the milk and the movement of some normal milk components out of the alveolar lumen into the perivascular space (Harmon, 1994). Theoretically,
all changes in mammary secretion during inflammation might be used to measure the effects of mastitis, but problems of instrumentation and standardization have prevented farm application of most tests. In dairy herds, mastitis is a production disease of major importance. Cows with clinical signs of mastitis are easily spotted by farmers and proper treatment can be applied. However, subclinical infections may not be observed and remain untreated. Cow-side tests, such as the California Mastitis Test, are available but laboursome and time consuming if applied to a large number of animals (Petersen et al., 2004). For the screening of herds, the Somatic Cell Counts (SCC) are used despite the fact that high levels do not necessarily reflect mastitis (Salminen, 1995). In order to detect subclinical infections, APP might be applied. For a test being useful in routine investigations for mastitis, it is important that it can be applied to milk samples which are readily available than serum or plasma.

The first acute phase proteins measured from milk and used as indicators of inflammation are bovine serum Albumin and α1 anti-trypsin (Giesecke and Viljjoen, 1974; Sandholm et al., 1984). Also there is evidence that clinical mastitis can be revealed by high serum and milk concentration of haptoglobin and serum amyloid A (Eckersall et al., 2001). The C-reactive protein is not regarded as an acute phase protein in cattle (Eckersall and Conner, 1988), but has been tested as an indicator for mastitis. The concentration of C-reactive protein was shown to increase in bovine milk during mastitis (Schrodal et al., 1995). The capacity of the milk C-reactive protein to distinguish between healthy and mastitic quarters was found to be poor (Pyorala, 2003).

Fb is the coagulation factor I, acute phase protein and is produced more rapidly than degraded during the inflammation. Another important function of Fb is the formation of fibrin matrix that enables the movement of fibroblasts and other cells and stimulates their production during the healing of damaged tissue (Bakes and Illek, 2006). Fb specifically binds to CD11/CD18 integrins on the cell surface of migrated phagocytes, thereby triggering a cascade of intracellular signals that lead to enhancement of degranulation, phagocytosis, antibody-dependent cellular cytotoxicity and delay of apoptosis (Sirin et al., 1998; Ruble et al., 2001). Cp acts as an anti-inflammatory agent by reducing the number of neutrophils attaching to the endothelium and acting as an extra cellular scavenger of peroxide (Segelmark et al., 1997).

The mean concentration of plasma Fb in subclinical cases with healthy cows showed significant difference (p<0.01), but the mean concentration of plasma Cp between subclinical groups and healthy cows was not significant (p>0.05). This difference between two APP can be as a result of their discrepancy in blood as compared with inflammatory stages in cattle, so that Fb was taken major APP, but Cp is minor APP (Murata et al., 2004).

The mean milk Fb and Cp concentrations in mastitic groups were significantly different with healthy cows (p<0.01). It is probable that most serum proteins leak into milk across the blood-mammary barrier as a result of the disruption caused by the inflammation due to mastitis. However, there have been reports of the extra hepatic synthesis of acute phase proteins (Eckersall et al., 2001). Although the acute phase proteins have been conventionally thought to be synthesized in the liver, there have been reports of the expression of the messenger RNA for these proteins during the acute phase response in extra hepatic tissues such as lung (Yang et al., 1995), intestinal epithelium (Vreugdenhil et al., 1999) and endometrium (Timmer and Schultz, 1987). For example, Cp is synthesized primarily in the liver but is also induced at extra hepatic sites (Pan et al., 1996; Mazumder et al., 1997). There is considerable potential for the use of a biological marker. Such as an acute phase protein, which is present in milk and can be measured routinely and reliably, for the objective and early diagnosis of mastitis. Such a marker could be particularly important for the continued development of robotic milking systems in which the manual examination of milk and cows is not practicable (Mottram, 1997). It might also provide a more accurate and earlier diagnosis of intramammary infection, reducing the time to treatment and thus possibly reducing the adverse effects of mastitis in both economic and welfare terms.

The results of this study show that Fb and Cp can be detected and quantified in milk from dairy cows with mastitis; the technique could have major implications for the diagnosis and treatment of this important disease. Plasma Fb for diagnosis of subclinical mastitis was suitable, but plasma Cp cannot be detected from dairy cows with subclinical mastitis.

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