MICROBIOLOGICAL AND SYSTEMIC IMMUNOLOGICAL CHANGES IN COWS WITH CLINICAL MASTITIS

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Summary
Mastitis is one of the most important bovine diseases causing economic losses to dairy producers. The research aimed to monitor microbiological and related systemic immunological changes in milking cows raised under semi-intensive conditions, showing signs of mastitis in one or more quarters of the udder, to potentially improve the treatment and increase the number of healed animals. Bacteriological (cultivation and API biochemical identification) and immunological (zinc precipitation technique for total IgG, PEG 4.2% precipitation to quantify circulating immune complexes, carbon particle inclusion test for phagocytosis and in vitro blast transformation technique for leukocyte functions) test were used. Extracts of Echinacea angustifolia, Hippophae rhamnoides, Silybum marianum, Aloe vera and Thymus vulgaris were tested in vitro for their restoring potential of adaptive cell mediated immunity. Microbiological results indicated the prevalence of Gram positive rods, but only one strain of S. aureus was identified (6.6%), the rest of the strains being classified as S. sciuri, S. xylosus and S. lentus. The blood levels of total Ig were statistically significantly increased (0.079-0.090 ODU, p<0.01-0.001) when compared to those in healthy animals, indicating the increase of systemic humoral response to local antigenic stimulation. CIC values did not exceed the physiological limit (0.004±0.0004 ODU). The phagocytic activity at systemic level was very week (0.01±0.008 to 0.05±0.004) when compared to that observed under physiological circumstances. Stimulation indices in control cultures were lower than normal (36.97±10.03%) as well as the response to the clasical mitogen PHA M. The most effective extracts were the Thymus vulgaris and Aloe vera ones, with a significant stimulatig effect over the untreated control culture (+59.43%, p<0.05 and +49.58%, p<0.05, respectively). The data suggested an impeded systemic response during a localised infection, meaning an increased susceptibility to other potential intercurrent pathogens. The tested vegetal extracts could be of help in increasing systemic immunity.

Key words: cows, mastitis, microbiology, systemic immunology

Introduction
Mastitis (mammary gland inflammation) is one of the most important bovine diseases causing economic losses to dairy producers. Mammary gland inflammation is a consequence of the activity of a number of cell and soluble factors that function together to eliminate invading microorganisms (Oviedo-Boyso et al., 2007). However, the cellular and soluble immune components associated with mammary tissues and secretion also can play an important role in protecting the gland from infectious diseases, such as mastitis (Sordillo et al., 1997). Raw (unpasteurized) milk has been found to participate in spreading of illnesses caused by Listeria, Campylobacter, Yersinia, Salmonella and E. coli. Milk and other
dairy products are reported to be frequently infected with \textit{Staphylococcus aureus}. Also \textit{Streptococcus agalactiae} has been described as one of the most common agents of invasive infections (Karima et al., 2006).

With severe clinical mastitis, abnormalities of milk are easily observed and milk is discarded by the producer. Such milk normally would not enter the food chain. Still, expenses occasioned by treatments and preventive/control procedures increase the overall costs for milk production.

The research aimed to monitor microbiological and related systemic immunological changes in milking cows raised under semi-intensive conditions, showing signs of mastitis in or more quarters of the udder, to potentially improve the treatment and increase the number of healed animals.

**Material and methods**

The experiment was conducted on a semi-intensive milk farm, on cows showing clinical mastitis (n=25) of different lactations and ages. Milk and blood were sampled from each animal in sterile conditions and processed in the laboratory. The first few drops of milk were discarded and subsequently the samples were collected into sterile recipients. Blood was sampled on heparine (50 IU/ml) and subjected to blast transformation and phagocytic tests within maximum four hours after sampling. Non heparinised blood was used to obtain sera.

\textit{Methods}. Microbiological tests were performed on milk samples from each quarter of each animal. Customary culture media were used at first. Isolated colonies were then inseminated and tested as a 0.5 turbidity on McFarland scale (tube 1) suspension in saline on various specific culture media (McConkey, Chapmann and blood agar) to obtain isolated colonies. API biochemical tests (API 20E, Apistaph, API non E, etc.) were used for identification.

\textit{Immunoglobulin measurements (total Ig)}. Total immunoglobulin, known as opsonins, play an important role in the “first line of defense”, that is innate immunity, against aggressors. At a pH 7.4, the electric charge and colloidal stability of gamma globulins are lower than those of serum albumins. Thus, concentrations as low as 24 mg l$^{-1}$ of metal salts precipitate the immunoglobulin. A volume of 6.6 ml of serum was mixed with 193.4 ml of a 0.024\% barbital buffer zinc sulphate solution and allowed to precipitate for 30 min at room temperature (22–23°C). Optical density (ODU) was then read spectrophotometrically ($\lambda$=475 nm, $d$=0.5 cm).

\textit{Circulating immune complex (CIC) measurements}. Measurement of the level of circulating immune complexes allows evaluation of the molecular clearance capacity at a particular moment. Part of the collected blood was allowed to clot for 30 min at 37°C and then centrifuged at 1308 $\times$g for 10 min. Sera were removed and kept at $-20^\circ$C until tested. A 4.2\% polyethylene glycol (PEG) solution in borate buffer was used as the precipitating agent, while buffer-treated samples served as controls for borate-induced precipitation. The reaction was performed in a 96-well-plate to enhance spectophotometrical readings. Volumes of 196.7 $\mu$l of borate buffer and PEG solution, respectively, were mixed with 3.3 $\mu$l samples of the serum, for each sample, in parallel wells. The samples were allowed to precipitate at room temperature (22–23°C) for 60 min, then read spectrophotometrically at a wavelength of 450 nm in the test plate ($d$=0.5 cm) (multichannel spectropho-meter SUMAL PE2, Karl Zeiss, Jena, Germany). CIC concentrations, expressed in optical density units (ODU) were calculated by subtracting
the value of the control (serum + buffer) from that of the PEG precipitate.

**Carbon particle inclusion test.** Phagocytic cells engulf in vitro inert particles such as carbon as their ability to protect the organism from intruders. The measurement of this activity suggests their defensive capacity against real, such as microbial, assault. 50 microliter aliquots of heparinised blood were mixed with 2 microliters of supernatant of India ink, obtained by centrifugation at 6000 rpm for 40 min (Hettich centrifuge, Germany). 15 microliters of the mixture were immediately transferred to 2 ml of saline and the rest was incubated at 37 °C for 20 min. Another aliquot of the same size was transferred to saline and the incubation was continued up to 40 min, repeating the operation. All the tubes containing the saline, blood and ink mixture were centrifuged at 1800 rpm and the supernatants were subjected to spectrophotometrical readings (λ=535 nm, d=1 cm). The results were expressed as the ln of the slope/20 min (first and second incubation intervals).

**Leukocyte blast transformation test** measures the in vitro reactivity of mononuclear cells to sensitizing (in vivo encountered) antigens. Cell growth was quantified by means of glucose consumption technique. A sample (640 µl) of blood was diluted with four times the amount of RPMI 1640. The mixture was distributed in sterile 96 well-plates, 200µl/well. Eight variants were performed for each individual animal, namely (a) untreated control culture, (b) phytohaemagglutinin-M (PHA) (1 µl per well), (c-h) alcohol and alcoholic extracts of Echinacea angustifolia, Hippophae rhamnoides, Sylibum marianum, Aloe vera and Thymus vulgaris (1.5 µl per well) treated cultures. The cultures were incubated in a 5% CO₂ atmosphere for 72 hours at 37.5°C. Glucose concentrations were estimated in the starting medium and all the in vitro experimental variants, at the end of the incubation period, compared to a standard (100 mg/dl) glucose solution by means of an orto-toluidin colorimetric test. For that, 12.5 µl of the cultural supernatant were transferred to 0.5 ml of orto-toluidine reagent, boiled for 8 minutes, suddenly cooled and subjected to spectrophotometrical reading at 610 nm wavelength (SUMAL PE2, Karl Zais, Jena, Germany), using the reagent as a blank. Transformation index was calculated as follows: Tİ%= (MG-SG)/MG x 100, where Tİ= blast transformation index, MG=glucose concentration in the starting culture medium, SG=glucose concentration in the sample after the incubation period.

Mean values, standard deviations and the statistical significance of the differences between the vegetal extract treatments and against controls were calculated.

**Results and discussion**

The overall impact of mastitis on the quality and quantity of milk produced for human consumption has provided the impetus to better understand the pathophysiology of the mammary gland and develop ways to enhance disease resistance through immune regulation. As such, the bovine species has played a critical and prominent role in our current understanding of mammary gland immunobiology (Sordillo and Streicher, 2002).

Microbiological results indicated the prevalence of Gram positive rods, such as *Staphylococcus*, 15 of the tested samples being positive on Chapmann culture medium and by API Staph biochemical test. Nevertheless, only one strain of *S. aureus* was identified (6.6%), the rest of the strains being classified as *S. sciuri*, *S. xylosus* and *S. lentus*. The results did not incriminate streptococci or Gram negative rods in the etiology of the mastitis on the investigated farm.
Technological advances in immunology have led to the availability of new research tools that can facilitate the study of mammary gland immunity and disease pathogenesis. In recent years, considerable research effort has focused on enhancing the natural defense mechanisms of the mammary gland during periods of heightened susceptibility to disease (Sordillo et al., 1997). It is well known that bacterial, cow and environmental factors are interdependent and influence mastitis susceptibility (Burvenich et al., 2003).

Most researchers now accept that the PMN is a key factor in the cows' defense against intramammary infection with E. coli. Effective elimination of the pathogen by neutrophils is important for the resolution of infection and the outcome of mastitis (Burvenich et al., 2003; Wenz et al., 2006). While PMN are phagocytosing and destroying the invading pathogens (Zecconi et al., 1994), they inadvertently release chemical mediators which induces swelling of secretory epithelium cytoplasm, sloughing of secretory cells, and decreased.

However, also innate immunity has to function and develop in time, depending on the lactation cycle, and its behavior and evolution in time in such a dynamical system is a challenge and a problem at the same. The defense of mammary gland is characterized by its complexity and over the last years many data show that there are tight connections with the mononuclear cells in mammary gland tissue. Today it is known that T cells play a central role in orchestrating the immune response (Rainard and Riollet, 2006).

Total Ig content quantification during the clinical course of the mastitis could indicate the functional status of opsonins, and implicitly the level of humoral protection. The results were shown in table 1.

In dairy cows with signs of clinical mastitis, the blood levels of total Ig were statistically significantly increased (0.079-0.090 ODU, p<0.01-0.001) when compared to those in healthy animals (Vasiu et al., 1995), indicating the increase of systemic humoral response to local antigenic stimulation. CIC values did not exceed the physiological limit (0.003-0.015 ODU). Thus, the clearance of CIC when total Ig increased, was preserved at normal levels.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Circulating immune complexes</th>
<th>Total Ig</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>PEG</td>
<td>TB</td>
</tr>
<tr>
<td>Mean</td>
<td>0.025</td>
<td>0.021</td>
</tr>
<tr>
<td>Stddev</td>
<td>0.0055</td>
<td>0.0055</td>
</tr>
</tbody>
</table>

In the lactating bovine mammary gland, the innate immune system plays a critical role in determining the outcome of these infections. Neutrophils are key effector cells of the innate immune response to infection, and their function is influenced by many physiological events that occur during the transition period (Rainard and Riollet, 2006).

Opportunistic infections occur when the integrity of the host immune system is compromised by physical and physiological conditions that make the host more susceptible. Their defense system is unable to modulate the complex network of innate immune responses, leading to incomplete resolution of the pathogen and the inflammatory reaction (Oviedo-Boyso et al., 2007).
Table 2. Phagocytic activity for the first and second reading periods

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ln 0 min</th>
<th>Ln 20 min</th>
<th>Ln 40 min</th>
<th>Ln 0-20 min</th>
<th>Ln 20-40 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>-0.78</td>
<td>-0.754</td>
<td>-0.761</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>Stdev</td>
<td>-2.41</td>
<td>0.2457</td>
<td>0.1889</td>
<td>0.008</td>
<td>0.004</td>
</tr>
</tbody>
</table>

The phagocytic activity at systemic level (table 2) was almost constant, but very week when compared to that observed under physiological circumstances (0.135 ± 0.065) in this species. The slower phagocytosis during the first period might suggest the adjustment of the cells to the in vitro conditions, leading to increased activity during the second incubation period.

The in vitro leukococyte blast transformation indices were presented in table 3. Men values in control cultures were lower than normal (54.31±3.54 %, Vasiu et al., 1995) as well as the response to the clasical mitogen PHA M, that showed a slight increase (+26.24%) above the control stimulation index. All extracts exerted a stimulating effect on the in vitro blastogenesis, when compared to control cultures, suggesting their potential use in stimulating the systemic immunity in mastitic cows. In this experiment, the most effective were the *Thymus vulgaris* and *Aloe vera* extracts, with a significant stimulatig effect over the untreated control culture (+59.43%, p<0.05 and +49.58%, p<0.05, respectively).

Table 3. Blast transformation indices (%)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>PHA M</th>
<th>Alcohol</th>
<th>Echinacea</th>
<th>Hippophae</th>
<th>Sylibum</th>
<th>Aloe</th>
<th>Thymus</th>
</tr>
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<tbody>
<tr>
<td>Mean</td>
<td>36.97</td>
<td>46.67</td>
<td>55.00</td>
<td>51.36</td>
<td>52.27</td>
<td>46.36</td>
<td>55.3</td>
<td>58.94</td>
</tr>
<tr>
<td>Stdev</td>
<td>10.03</td>
<td>7.94</td>
<td>12.18</td>
<td>8.79</td>
<td>9.21</td>
<td>9.71</td>
<td>8.65</td>
<td>6.81</td>
</tr>
</tbody>
</table>

Legend: PHA M= phytohemagglutinin, Echinacea=Echinacea angustifolia, Seabuchthorn=Hipopphae rhamnoides, Sylibum=Sylibum marianum, Aloe=Aloe vera, Thymus=Thymus vulgaris

The results indicated the general involvement of the immune system in the development of a local infection such as mastitis in cows.

Conclusions

The overall results of the investigation indicated a dominance of *Staphylococcus* spp., (*S. aureus*-one strain, *S. xylosus*, *S. lentus* and *S. sciuri*), combined with an increased systemic level of total Ig, normal levels of CIC, decreased systemic phagocytosis and leukocyte blast transformation. The data suggested an impeded systemic response during a localised infection, meaning an increased susceptibility to other potential intercurrent pathogens. The tested vegetal extracts (*Echinacea angustifolia*, *Hippophae rhamnoides*, *Sylibum marianum*, *Aloe vera* and *Thymus vulgaris*) could be of help in increasing systemic immunity, most efficient being those of *Aloe vera* and *Thymus vulgaris*. Investigations carried out on the changes in systemic immunity in relation to the local infection could suggest pathways to improved treatment and enhanced healing of the diseased animals, diminishing the economic damage and health risk for consumers.
References


