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ORIGINAL ARTICLE

In vitro differential neutrophil phagocytosis activity on *Staphylococcus aureus* when obtained from blood and milk of dairy cows in early lactation period

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ABSTRACT. The ability of bovine blood and milk neutrophils (PMN) to phagocytize fluorescein conjugated *Staphylococcus aureus* was assessed in 14 clinically healthy dairy cows in early lactation period during 4-6 weeks. Hypotonic erythrocyte lysis (HEL) was used for granulocyte cell isolation from blood and increased leukocyte concentration from milk was induced by intramammary perfusion of *Escherichia coli* 0:26:B6 lypopolysaccharide B (LPS). A greater *in vitro* mean phagocytosis index 7.34 ± 1.55 was observed in blood PMN in comparison to milk cells 4.71 ± 1.31 ($p < 0.001$). PMN phagocytosis ability in blood was 86.5 ± 9.6%, compared to reduced milk cell ability ($P < 0.01$). The linear relationship between blood and milk cell phagocytosis was $y = 0.84 x + 3.37$ ($R^2 = 0.3783$) with association of cell phagocytosis activity ($r = 0.615$). It is concluded that PMN concentration cells obtained by erythrocyte lysis and LPS udder stimulation are useful for *in vitro* reproducible phagocytosis assays. These findings are consistent with mammary gland response which induced rapid migration of blood cells. The procedure may facilitate the evaluation of early innate immune response against intramammary bacterial infections in dairy cows.

Key words: Dairy cows, neutrophils, *in vitro* phagocytosis, blood and milk *Staphylococcus aureus*.

RESUMEN. Se evaluó la diferencia entre los neutrófilos (PMN) de sangre y leche para fagocitar *in vitro Staphylococcus aureus* marcados con fluoresceína en 14 vacas lecheras clínicamente sanas durante 4-6 semanas. La concentración de PMN de sangre se realizó por lisis hipotónica de eritrocitos (HEL) y en leche mediante perfusión intramamaria de lipopolisacárido B de *Escherichia coli* 0:26:B6 (LPS). El índice de fagocitosis *in vitro* fue mayor en los PMN de sangre 7.34 ± 1.55 comparados con las células en leche 4.71 ± 1.31 ($P < 0.001$). La capacidad de fagocitosis de los PMN de sangre fue 86.5 ± 9.6%, comparada con una capacidad reducida de las células en leche ($P < 0.01$). Se observó una relación lineal en la actividad de fagocitosis de las células de sangre y leche ($y = 0.84 x + 3.37$; $R^2 = 0.3783$), con una asociación ($r = 0.615$). La concentración de PMN es útil y reproducible para ensayos de fagocitosis *in vitro*. Los hallazgos son consistentes con la capacidad de respuesta de la glándula mamaria, al inducir una rápida migración de PMN de la sangre frente a la infección. El procedimiento descrito puede facilitar la evaluación de la respuesta inicial de la respuesta inmune innata en las vacas lecheras.

Palabras clave: Vacas lecheras, neutrófilos, fagocitosis *in vitro*, sangre y leche *Staphylococcus aureus*.

INTRODUCTION

Neutrophils are part of the innate defense cell mechanism in mammals which use phagocytosis and intracellular oxygen microbicide action (Bochsler and Slauson, 2002). During the early infection by *Staphylococcus aureus* in milking cows, a considerable increase in somatic cell counts is produced, with a high proportion of PMN (Kelly et al., 2000; Peller et al., 2003).

The severity of the infection produced by *S. aureus* as well as the clinical evolution of mastitis considerably affect PMN functional activity in the mammary gland (Tomita et al., 2000). Mammary gland infection persistence as well as high somatic cell counts (SCC) indicate antibody presence against it in milk (Barrio et al., 2003; de Haas et al., 2002; Younis et al., 2003). It is possible that the subclinical infection is related to the intracellular survival of *S. aureus* (Kerro Dego et al., 2002).

During chronic infections there is low bactericidal activity. There is evidence that suggests that the functional PMN activity as well as its proportion in milk, could make the mammary gland susceptible to *S. aureus* infection (Piccinini et al., 1999; Dosogne et al., 2003). In the last decades, dairy herd reproduction has considered genetic selection of milking cows directed towards a higher milk production and as consequence an increase in the susceptibility to mastitis in high production cows (Kearney et al., 2004; Ikonen et al., 2004). In contrast, cows with low SCC and an efficient phagocytosis activity during the milking period show a better immune response as natural resis-

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tance in the mammary gland (Dekkers et al., 1994; de Haas et al., 2004). This could be considered as a selective strategical genetic factor tending to reduce mastitis in milking herds (Dorp et al., 1998; Odegard et al., 2003).

The study of PMN functioning in cattle is important for innate immune cell evaluation as it is the first line immune defense in hosts against bacterial infections (Leitner et al., 2000; Kehrli and Shuster, 1994).

Blood and milk are available sources for phagocytic cells in cows (Smits et al., 1997; Guidry et al., 1998). *In vitro* phagocytosis assays with PMN from blood cells require rapid purification methods to obtain neutrophils and other leukocytes (Zwahlen and Roth, 1990). Different isolation methods for granulocytic leukocytes from peripheral blood have been previously described; such as dextran sedimentation (Solberg, 1972), cold water erythrocyte lysis and cell resuspension in sodium saline solution (Carlson and Kaneko,1973), gradient concentration using polyvinilpyrrolidone coated silica particles (Pertoft et al., 1978), erythrocyte lysis and separation of cells in Ficoll-Hypaque (Roth and Kaeberle, 1981), hypotonic phosphate erythrocyte lysis (Field et al., 1985) Ficoll-Hypaque flotation and adherence chromatography (Hurley and Finkelstein,1986), metrizamide gradients (Hallén-Sandgren and Björok, 1988) and resuspended cells layered on Percoll (Kremer et al., 1992). Neutrophils from milk have been concentrated in lactating cows from udder by LPS leukocyte induced migration (Sanchez et al., 1994). These methods are selected depending on laboratory facilities and the expected *in vitro* PMN cell phagocytosis studies of leukocyte cells in cattle.

The objectives of this paper were to evaluate the *in vitro* differential phagocytosis activity (PHA) of bovine PMN cells in small samples from blood and milk in dairy cows as well as to describe the hypotonic erythrocyte lyses method used to obtain PMN from blood for *in vitro* assays for the evaluation of the first line immune defense in cattle.

MATERIALS AND METHODS

Dairy cows group

Fourteen clinically healthy dairy Holstein cows with two parturitions, in early lactation during 4 to 6 weeks were used. Cows used were selected based on the inclusion criteria of less than 200,000 somatic cells/ ml in milk samples. Estimated cell counts were carried out using a Coulter Counter ZM4 (Coulter Electronics LTD, Luton, England), according to the International Dairy Federation procedures (1984).

Mammary glands were considered as uninfected by *S. aureus* after three negative consecutive isolations in different sampling periods. Milk samples were aseptically collected from the animals and evaluated by routinely bacteriological procedures (Boerlingin et al., 2003; Capuco et al., 1986).

Blood and milk polymorphonuclear leukocyte isolation

Blood was collected from jugular vein, using vacuum sample tubes containing citric acid dextrose solution 1:9 (Worku et al., 1994). The use and care of the dairy cows in this study was supervised following institutional animal care procedures and husbandry committee.

Leukocyte total counts were performed by hematological methods using hemocytometer (Hausser Scientific, Horsham, PA, USA) and to determine differential leukocyte counts blood glass smears Giemsa stained were used.

PMN cells were obtained from blood centrifuged at 1,000 g for 20 min at 4 ºC in propylene conical tubes. Plasma and buffycoat with 1/4 erythrocyte column were discarded. Erythrocytes were lysed in the remaining sample using the following procedures: 4 ml of blood were added to a sample tube containing 8 ml of cold 4 ºC saline A solution (NaCl, 0.020 M), after 30 seconds 2 ml of cold saline B solution (NaCl 0.164 M) were used to restore isotonicity. The lysate was centrifuged three minutes at 300 g and the recovered cell pellet was washed twice in Hank's balanced solution (0.1% of bovine serum albumin Ca^{2+} and Mg^{2+} free), using the same procedure. Pellet was resuspended in supplemented RPMI 1640 cell culture media (15% v/v fetal calf serum inactivated at 56 ºC, penicillin 100 UI/ml, streptomycin 100 µg/ml, L-glutamine 4 mM, and pyruvate 30 µg/ml). PMN package was adjusted to 5 x 10^6 viable cells/ml, with viability of 95% estimated by trypan blue exclusion and 95% purity of mature neutrophils on slide films Giemsa stained (Lyn et al., 1995). Samples contaminated with mononuclear cells required an additional concentration procedure: cell suspension was layered on 3 ml of lymphocyte separation solution Lymphoprep (Nycomed Pharma AS, Oslo, Norway), which was centrifuged at 1,500 g washing the pellet twice with Hank's balanced solution.

PMN from milk, were obtained after intramammary infusion of 0.3 µg *Escherichia coli* O:26:B6 lipopolysaccharide (Sigma Aldrich, Saint Louis, Mo, USA) in 3 ml phosphate buffered solution pH 7.2 sterile filtered by 0.25 µm acetate membrane. 100 ml milk samples were taken fourteen hours later by hand milking in a propylene container with EDTA 20:1 (milk:EDTA disodium salt solution 0.15 M). PMN cells were concentrated by centrifugation at 1,800 g for 20 min at 4 ºC. Cell pellet was washed three times with balanced Ca^{2+} and Mg^{2+} salt free Hank's solution, resuspended in supplemented RPMI-1640, with a final concentration of PMN of 5 x 10^6 cells/ml.

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Polymorphonuclear leukocyte in vitro *phagocytosis*

In vitro phagocytosis assay was conducted 1:10 (neutrophil:bacteria), using the PMN cell suspension (2.5 x 10^6 cells/ml), obtained from blood and milk. *S. aureus* Newbold 304 strain, ATCC 29740 (American Type Cell Collection, Manassas, VA, USA) bacterial suspension was heat inactivated and adjusted to 2 x 10^8 CFU/ml (0.5 OD at 550 nm) and conjugated with fluorescein isothiocyanate (Sutra et al., 1990).

In vitro phagocytosis assay was performed with 0.1 ml of bacterial suspension and 0.1 ml of autologous chemotactically inactive serum added to 0.8 ml neutrophil suspension in polyethylene microtubes. For milk neutrophils, autologous whey milk was used in the assay, instead of serum. Positive and negative controls were used in the phagocytosis assay.

Samples were incubated one hour at 37 ºC in oscillating bath at 20-25 cycles/min. Preparations were lysostaphin treated (1 µg/ml, Sigma), reincubated for 30 minutes, centrifuged and washed twice in cold RPMI 1640 cell culture media. Glass slide preparations were observed using ultraviolet light microscope. Phagocytosis index (PHI) was estimated as the mean number of phagocyted *S. aureus* uptake per neutrophil, counting 200 PMN cells in different microscope fields by triplicate assays. Phagocytosis ability (PHC) was considered as the percentage of phagocyting neutrophils by microscope field.

Statistical analyses

Statistics was performed by randomized design using a fixed effects statistical model, where the cow was the random term, sample source the fixed term, and blood and milk neutrophil counts at time of sampling and actual day of lactation used as covariates, parturition and purification protocol as fixed effects. Results of the differences between control and experimental groups were analyzed by one factor ANOVA and regression analysis ($p < 0.05$), using the application software Megastat (version 9.1b. distributed by Mc Graw-Hill as a complement of Microsoft Office®, Excel 2003 Microsoft, Mexico).

RESULTS

The values for dairy cow's blood were; erythrocyte package cell volume 38 ± 2.14% and leukocyte cell counts 8.80 ± 1.13 x 10^3 cells/µl. After final hemolysis cell suspension purity was 95.4 ± 0.8 % of mature PMN and cell viability was over 95%. Granulocytic recovered cells from blood did not have a tendency to clump. Just one sample, relatively contaminated with mononuclear cells after erythrocyte lysis required an additional concentration procedure. PMN leukocyte cells from milk samples were recovered as highly viable pure cells after centrifuge concentration.

PHI in blood neutrophils was significantly higher 7.34 ± 1.55 when compared to cells obtained from milk secretion 4.71 ± 1.13 ($P < 0.05$) (Table 1), explained by the regression equation $y = 0.8404x + 3.3737$, (R^2 0.3783). There was an association between polymorphonuclear cell phagocytosis index from blood and milk ($r = 0.615$). Blood PMN phagocytosis ability was significantly different 86.5 ± 9.6% compared to the reduced PHC in PMN from milk 74.3 ± 8.5 % ($P < 0.05$).

Mean *in vitro* PHI after PMN blood and milk exposure to *S. aureus* in cow groups according to individual data, indicate that some cows had more PMN cell PHA (Figure 1).

DISCUSSION

Different PHA was observed in PMN from blood and milk when *in vitro* phagocytosis activity was evaluated in milking cows in early lactation period.

The functional activity of bovine blood PMN was not affected according to the evaluated parameters in our study. Kampen et al., (2004) observed satisfactory functional phagocytosis in bovine polymorphonuclear leukocytes using a similar method.

The results indicate constant PHA in blood PMN, and less adverse effects derived from isolation procedures. The bovine blood PMN isolation protocol described is potentially useful for PMN *in vitro* phagocytosis assays (Kelm et al., 1997; Piccini et al., 1999).Our procedure may facilitate blood PMN phagocytosis evaluation of *S. aureus* infection mechanisms in cattle (Cooray and Hakansson, 1995; Dosogne et al., 2001; Vangroenweghe et al., 2001).

The procedure described above is simple and reproducible, with no significant statistical differences observed when compared to the gold standard (Carlson and Kaneko, 1973) with additional advantages such as inexpen-

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Table 1. *In vitro* phagocytosis index and phagocytosis ability of bovine polymorphonuclear leukocytes from dairy cows.

PMN cell source	PHI Phagocytosis index	PHC Phagocytosis ability
Blood	7.3 ± 1.5	86.5 ± 9.6
Milk	4.7 ± 1.3	74.3 ± 8.5
Overall	6.0 ± 1.8	80.4 ± 9.1

All data given as Mean ± S.E.M., *n*: 14 samples. PHI and PHC were significantly different according to PMN cell source ($P < 0.05$)

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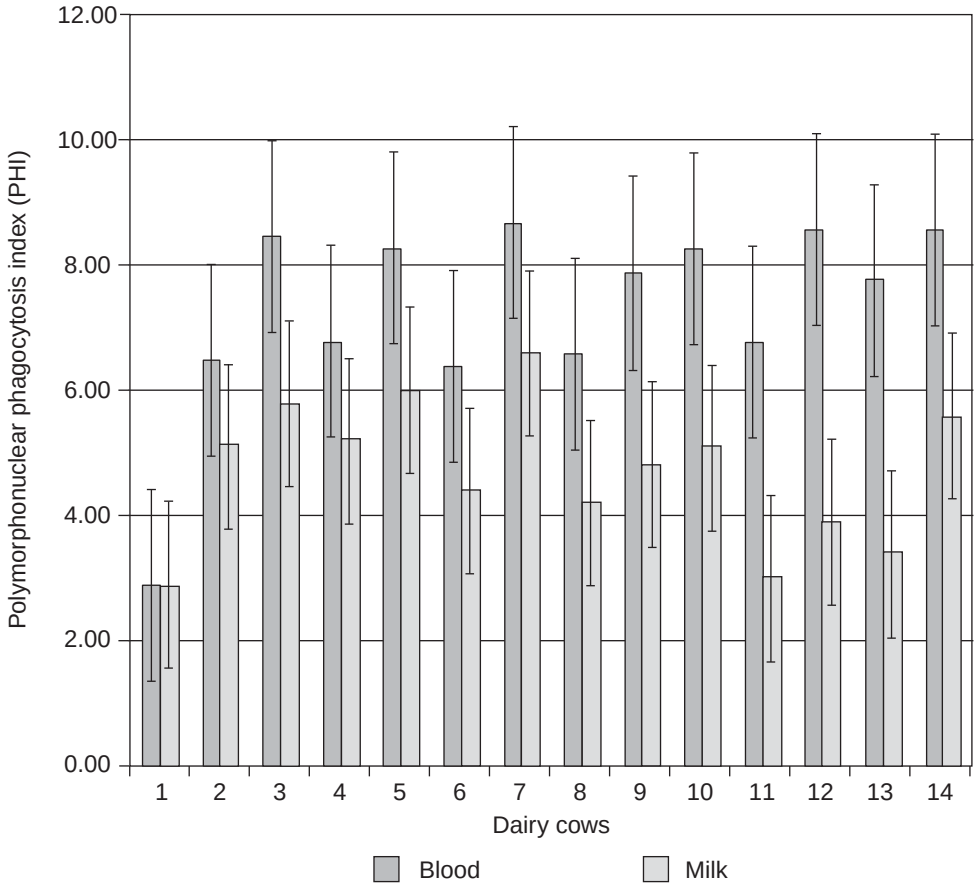

Individual data show increased PHI activity ranging from 2.90 to 8.90 in blood, and reduced values observed in milk (2.9 to 6.6) (P < 0.001).

Figure 1. In vitro *phagocytosis index of bovine blood polymorphonuclear leukocytes in the dairy cows.* n *14*

siveness and effective preparation of a rich fraction of PMN leukocytes.

Other erythrocyte lyses methods produce low PMN yields and cell pellets contamination with erythrocytes, as shown in bovine and porcine samples (Field et al., 1985). PMN yield concentration in samples depend on the proportion of granulocytic cells in blood of donor cows, neutrophil purity is high in healthy cows (Riding and Willadsen, 1981). There was no problem when eosinophils were present in samples, since they play a minor role in bacterial phagocytosis (Saad and Hagelton, 1985). The additional concentration procedure applied on samples after erythrocyte lysis, slightly increased neutrophil purity proportion with the same results observed by Hurley and Finkelslein (1986).

The reduced *in vitro* PMN milk phagocythosis observed in the assays of the cow groups studied, can be related to widely described milk components as well as the PMN life span in uninfected bovine mammary glands (Paape et al., 2000; Sladek et al., 2000; Dosogne et al., 2001).

We confirmed that LPS infused in mammary gland, increased the total number of mature neutrophils in milk samples by enriched leukocytosis migration without clinical adverse effects on dairy cows (Capuco et al., 1986; Mehrzard et al., 2001). In the early lactation stage of dairy cows, neutrophils are the predominant cells in lacteal secretion in healthy mammary glands (Rivas et al., 2001; van Oostveldt et al., 2001).

The differences found in PMN phagocytosis activity shown in this study makes PMN from blood a sustainable cell source for *in vitro* phagocytosis activity assays in dairy cattle. The PMN isolation technique here described has a suitable and simple protocol which can be used for processing small volumes of bovine blood and milk.

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REFERENCES

1. Barrio, M.B., P. Rainard & B. Poutrel. 2003. Milk complement and the opsonophagocytosis and killing of *Staphylococcus aureus* mastitis isolates by bovine neutrophils. Microb Pathog 34:1-9.
2. Boerlin, P., P. Kuhnert., D. Hüssy & M. Schaellibaum. 2003. Methods for identification of *Staphylococcus aureus* isolates in cases of bovine mastitis. J Clin Microbiol 41: 767-771.
3. Bochsler, P.N. & D.O. Slauson. 2002. Inflammation and repair of tissue. pp. 198-206. In D.O. Slauson & B.J. Cooper (Eds). Mechanisms of the disease: a textbook of comparative general pathology. Third edition. Mosby an affiliate of Elseviere Science. St. Louis, Missouri, USA.
4. Capuco, A.V., M.J., Paape & S.C. Nickerson. 1986. *In vitro* study of polymorphonuclear leukocyte damage to mammary tissue of lactating cows. Am J Vet Res 47:663-668.
5. Carlson, G.P & J.J. Kaneko. 1973. Isolation of leukocytes from bovine peripheral blood. Proc Soc Exp Biol Med 142:853-856.
6. Cooray, R & L. Hakansson. 1995. Defective polymorphonuclear neutrophil function in dairy cows showing enhanced susceptibility to intramammary infections. Zentralbl Veterinarmed B 42:625-632.
7. de Haas, Y., H.W. Barkema & R.F. Veerkamp. 2002. The effect of pathogen-specific clinical mastitis on the lactation curve for somatic cell count. J Dairy Sci 85:1314-1323.
8. de Haas, Y., R.F. Veerkamp., H.W. Barkema., Y.T. Grohn & Y. H Schukken. 2004. Associations between pathogen-specific cases of clinical mastitis and somatic cell count patterns. J Dairy Sci 87:95-105.
9. Dekkers, J.C.M., B.A. Mallard & K.E. Leslie. 1994. Genetic improvement of resistance to mastitis of dairy cattle with special emphasis on somatic cell count. J Dairy Sci 77:616-618.
10. Dorp, T.E.V., J.C.M. Dekkers., S.M. Martin & J.P.T.M. Noordhuizen. 1998. Genetic parameters of health disorders, and relationship with 305-day milk yield and conformation traits of registered Holstein cows. J Dairy Sci 81:2264-2270.
11. Dosogne, H., F. Vangroenweghe., B. Barrio., P. Rainard & C. Burvenich. 2001. Decreased number and bactericidal activity against *Staphylococcus aureus* of the resident cells in milk of dairy cows during early lactation. J Dairy Res 68:539-549.
12. Dosogne, H., F. Vangroenweghe., J. Mehrzad., A.M. Massart-Leen & C. Burvenich. 2003. Differential leukocyte count method for bovine low somatic cell count milk. J Dairy Sci 86:828-834.
13. Field, T.R., M.R. Williams & K.J. Bunch. 1985. An improved method for isolation of a neutrophil-rich fraction from porcine blood. Br Vet J 141:355-361.
14. Guidry, A.J., C.N. O´Brien & L.W. Douglass. 1998. A bovine mammary endothelial and epithelial cells culture model of the blood milk barrier. Can J Vet Res 62:117-121.
15. Hallén-Sandgren, C.H & I. Björk.1988. A rapid technique for the isolation of highly purified, functionally intact bovine neutrophilic granulocytes. Vet Immunol Immunopathol 18:81-94.
16. Hurley, W. L & E. Finkelstein. 1986. Bovine leukocytes in mammary secretions during involution surface proteins changes. Am J Vet Res 47:2418-2422.
17. International Dairy Federation. 1984. Recommended methods for somatic cell conting in milk. Bull Int Dairy Fed 168:19.
18. Ikonen, T., S. Morri., A.M. Tyriseva., O. Ruottinen & M. Ojala. 2004. Genetic and phenotypic correlations between milk coagulation properties, milk production traits, somatic cell count, casein content, and pH of milk. J Dairy Sci 87:458-467.
19. Kampen, A.H., T. Tollersrud., S. Larsen., J.A. Roth., D.E. Frank & A. Lund. 2004. Repeatability of flow cytometric and classical measurement of phagocytosis and respiratory burst in bovine polymorphonuclear leukocytes. Vet Immunol Immunopathol 97:105-114.
20. Kelly, A.L., D. Tiernan., C. O'Sullivan & P. Joyce. 2000. Correlation between bovine milk somatic cell count and polymorphonuclear leukocyte level for samples of bulk milk and milk from individual cows. J Dairy Sci 83:300-304.
21. Kearney, J.F., M.M. Schutz & P.J. Boettcher. 2004. Genotype x environment interaction for grazing vs. confinement. II. Health and reproduction traits. J Dairy Sci 87:510-516.
22. Kelm, S.C., J.C. Detilleux., A.E. Freeman., M. E. Kehrli Jr., A.B. Dietza., L.K. Fox., J.E. Butler., I. Kasckovics & D.H. Kelly. 1997. Genetic association between parameters of innate immunity and measures of mastitis in periparturient Holstein cattle. J Dairy Sci 80:1767-1775.
23. Kerro Dego, O., J. E. van Dijk & H. Nederbragt. 2002. Factors involved in the early pathogenesis of bovine *Staphylococcus aureus* mastitis with emphasis on bacterial adhesion and invasion. A review. Vet Q 24:181-198.
24. Kherli, M.E & D.E. Shuster. 1994. Factors affecting milk somatic cells and their role in health of the bovine mammary gland. J Dairy Sci 77:619-627.
25. Kremer, W.D., E.N. Noordhuizen-Stassen., P.A. Henricks & H. van der Vliet. 1992. A procedure for parallel isolation of white blood cells, granulocyte and purified neutrophil suspensions from the peripheral blood of cattle. Vet Immunol Immunopathol 31:189-193.
26. Leitner, G., E. Shoshani., O. Krifucks., M. Chaffer & A. Saran. 2000. Milk leukocyte population patterns in bovine udder infection of different aetiology. J Vet Med B Infect Dis Vet Public Health 47:581-589.
27. Lin, Y., L. Xia., J.D. Turner & X. Zhao. 1995. Morphological observation of neutrophil diapedesis across bovine mammary gland epithelium *in vitro*. Am J Vet Res 56:203-207.
28. Mehrzard, J., H. Dosogne., E. Meyer & C. Burvernich. 2001. Local and systemic effects of endotoxin mastitis on the chemiluminescence of milk and blood neutrophils in dairy cows. Vet Res 32:131-144.
29. Odegard, J., G. Klemetsdal & B. Heringstad. 2003. Genetic improvement of mastitis resistance: validation of somatic cell score and clinical mastitis as selection criteria. J Dairy Sci 86:4129-4136.
30. Paape, M.J., K. Shafer-Weaver., A.V. Capuco., K. van Oostveldt & C. Burvenich. 2000. Immune surveillance of mammary tissue by phagocytic cells. Adv Exp Med Biol 480:259-277.
31. Peeler. E.J., M.J. Green, J.L. Fitzpatrick, & L.E. Green. 2003. The association between quarter somatic-cell counts and clinical mastitis in three British dairy herds. Prev Vet Med 59:169-180.
32. Pertoft, H., T.C Laurent., T. Laas & L. Kagedal. 1978. Density gradient prepared from colloidal silica particles coated by polyvinyl pyrrolidone (Percoll). Anal Biochem 88:271-272.
33. Piccinini, R., V. Bronzo., P. Moroni., C. Luzzago & A. Zecconi. 1999. Study on relationship between milk immune factors and *Staphylococcus aureus* intramammary infections in dairy cows. J Dairy Res 66:501-510.
34. Riding, G.A & P. Willadsen. 1981. Simultaneous isolation of bovine eosinophils and neutrophils on gradients of Percoll. J Immunol Methods 46:113-119.
35. Rivas, A.L., F.W. Quimby., J. Blue & O. Cksaygan. 2001. Longitudinal evaluation of bovine mammary gland health status by somatic cell counting, flow cytometry and cytology. J Vet Diagn Invest 13:399 - 407.
36. Roth, J.A & M.L. Kaeberle. 1981. Isolation of neutrophils and eosinophils from peripheral blood of cattle and comparison of their functional activities. J Immunol Methods. 45:153-164.

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37. Saad, M.A & M. Hageltorn. 1985. Flow cytometric characterization of bovine blood neutrophil phagocytosis of fluorescent bacteria and zymosan particles. Acta Vet Scand. 26:289-307.
38. Sanchez, M.S., C. Ford & R.J. Yancey Jr. 1994. Effect of tumor necrosis factor- alpha, interleukin- beta, and antibiotics on the killing of intracellular *Staphylococcus aureus.* J Dairy Sci 77:1251-1258.
39. Solberg, G.O. 1972. Enhanced susceptibility to infection. A new method for the evaluation of granulocyte functions. Acta Pathol Microbiol Scan [B] Microbiol Immunol 80:10-18.
40. Sladek, Z & D. Rysanek. 2000. Morphology of apoptosis of polymorphonuclear leukocytes isolated from mammary glands of unbread heifers. Vet Med Czech 3: 71-81.
41. Smits, E., C. Burvenich & R. Heyneman. 1997. Simultaneous flow cytometric measurement of phagocytotic and oxidative burst activity of polymorphonuclear leucocytes in whole bovine blood. Vet Immol Immunopathol 56:259-269.
42. Sutra, L., P. Rainard. & B. Poutrel.1990. Phagocytosis of mastitis isolates of *Staphylococcus aureus* and expression of type 5 capsular polysaccharide are influenced by growth in the presence of milk. J Clin Microbiol 28:2253-2258.
43. Tomita, G.M., Y. Wang., M.J. Paape., B. Poutrel & P. Rainard. 2000. Influence of bispecific antibodies on *in vitro* bactericidal activity of bovine neutrophils against *Staphylococcus aureus.* J Dairy Sci 83:2269-2275.
44. Vangroenweghe, F., H. Dosogne., J. Mehrzad & C. Burvenich. 2001. Effect of milk sampling techniques on milk composition, bacterial contamination, viability and functions of resident cells in milk. Vet Res 32:565-579.
45. van Oostveldt, K., F. Vangroenweghe., H. Dosogne & C. Burvenich. 2001. Apoptosis and necrosis of blood and milk polymorphonuclear leukocytes in early and mild lactating healthy cows. Vet Res 32:617-622.
46. Worku, M., M.J. Paape & W.W. Marquardt. 1994. Modulation of Fc receptors for IgG on bovine polymorphonuclear neutrophils by interferon gamma though de novo RNA transcription and protein synthesis. Am J Vet Res 55:234-238.
47. Younis, A., O. Krifucks., E.D. Heller., Z, Samra., A. Glickman., A. Saran G. Leitner 2003. *Staphylococcus aureus* exosecretions and bovine mastitis. J Vet Med B Infect Dis Vet Public Health 50:1-7.
48. Zwahlen, R.D & D.R. Roth.1990. Chemotactic competence of neutrophils from neonatal calves. Functional comparison with neutrophil from adult cattle. Inflammation. 14:109-123.

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