*Original article*

# IN VITRO ASSAY FOR PHAGOCYTIC ACTIVITY BY CANINE NEUTROPHILS FOR *Escherichia coli*

**A.P.Liyanage[1], M.R.C.K. Mallawa[2]** and **I.D. Silva[2]**

*1 Department of Animal Production and Health, Sri Lanka*
*2 Veterinary Teaching Hospital, University of Peradeniya, Sri Lanka*

**SUMMARY:** This study investigates the *in vitro* phagocytic activity of blood neutrophils of dogs in Sri Lanka for *Escherichia coli*. The study sample comprised of 70% mongrel and 30% cross bred dogs of both sexes. The leukocyte isolation method described yielded up to 12,930/µl viable leukocytes of which 61-90% was neutrophils, with viability ranging from 90-99% (mean 96%). The mean percent phagocytosis (MPP) of canine blood neutrophils was significantly higher at 30 minutes incubation (89%) than at 15 minutes incubation (70 %) with *E.coli*. The findings support the fact that innate immunity can be measured qualitatively and quantitatively by the technique described which will be useful for the identification of immunocompromised dogs.

## INTRODUCTION

The Innate immunity is the first line of defense in the body which includes physical and chemical barriers; circulating effector cells [phagocytes and natural killer cells (NK)], complement proteins and cytokines (Abba, 2007). Intact skin and its secretions are impermeable to most infectious agents and act as major physical barriers generating the first line of defense. Those microorganisms that evade the epithelial barrier get killed by natural antimicrobial peptides, but many get engulfed and killed by phagocytes (Tizard, 2009; Roitt, 1988). Phagocytosis is an active process in phagocytic leukocytes, namely, neutrophils, eosinophils, monocytes, and macrophages. Neutrophils are the first responders that phagocytize and kill invading pathogens (Abba, 2007). Phagocytosis of cytokine activated neutrophils and their microbicidal activities are the first line of cellular defense against invading pathogens, and are a major part of innate immunity (Silva *et al*. 1988).

Phagocytic activity is a continuous process involving discrete stages occurring in a sequential manner, namely, cell activation, chemotaxis, adherence, ingestion, and destruction (Tizard, 2009). The factors influencing phagocytic properties of cells include cell age, energy (ATP), and integrity of cellular skeleton such as microfilaments and microtubules (Jain, 1986). The important properties of bacteria influencing phagocytosis are surface charges, cell wall composition, capsule, and toxins. The environmental factors required are presence of opsonins, temperature (37- 40°C), pH (6-8), osmolarity, ionic composition and interfacial tension. To evaluate the efficiency of the phagocyte system, it is necessary to examine the number of phagocytes, their adhesion, chemotaxis, phagocytosis, and intra cellular killing mechanisms

(Gershwin *et al*., 1995). A deficiency or a malfunction at any stage of neutrophil function can impair the ability of an animal to overcome an infection (Gershwin *et al*., 1995). Severe sepsis in diseases may alter innate immune defenses, disrupt microbial clearance mechanisms, and complicate the pathophysiology of sepsis. Septic shock itself has significant effects upon the innate and adaptive host immune responses contributing to a state of sepsis-induced immune dysregulation (Opal and Cross, 2005). Although congenital or inherited immunodeficiency disorders are uncommon in the dog, immunodeficiency secondary to a wide range of causes (e.g., age, drug therapy, and chronic neoplastic or infectious disease) is relatively common in adult dogs. Most canine immune deficiencies are breed-related. Aged Beagle dogs have decreased neutrophil phagocytosis and neutrophil-related gene expression compared to younger dogs (Hall *et al*., 2010)

Neutrophil phagocytosis can be measured by several methods by allowing bacteria, yeast, or other particles and neutrophils to interact, and then to measure the number of neutrophils that have ingested particles, as well as the phagocytic index determined by the average number of organisms within a phagocyte (Kaneko and Harvey, 1997; Gershwin *et al*., 1995). A phagocytosis assay of canine blood neutrophils for bacteria is a long felt need to identify immune compromised dogs. The objective of this research was to establish an *in vitro* phagocytic assay for blood neutrophils isolated from clinically healthy dogs.

## MATERIALS AND METHOD

### Study group

Blood was drawn via cephalic venepunture into an EDTA tube and a tube without anticoagulant from

clinically healthy dogs of 2-10 years of age presented to Veterinary Teaching Hospital (VTH), University of Peradeniya. The study sample comprised of 70% mongrel and 30% cross bred dogs of both sexes. The EDTA blood was subjected to full blood count using MS 9-5V Veterinary Hematological counter (Melet Schloesing Laboratories, 2003 France) and the differential leukocyte counts were calculated from Leishman stained thin blood smears. Thirty six dogs with cellular parameters within the normal ranges and negative for blood parasites were selected for the study (Silva and Mallawa, 2010). Blood sample without EDTA was used to obtain fresh serum.

### Isolation of neutrophils

The leukocyte isolation protocol of Carlson and Kaneko (1973), which was modified by increasing the duration for RBC lysis to 50 seconds, based on results of a prior experiment done to measure the minimum time required to lyse RBCs in canine blood. The EDTA blood was centrifuged at 200 x g for 10 min at $4^{0}$C in a Hettich Universal 32 R centrifuge (Germany). The plasma and the buffy coat were decanted, in order to increase the harvest of phagocytes which were lodged in the hematocrit, based on preparatory investigations. The remaining packed red cells were mixed with 6ml of sterile Distilled Water (DW) pH 7.2 for 50 sec to lyse the RBCs. Then 4 ml of sterile phosphate buffered saline in 0.0132 M phosphate buffer with 2.7% NaCl, pH 7.2 (PBSb) was added to the cell suspension to restore isotonicity. Tubes were then mixed gently and centrifuged at 200 x g for 10 min. at $4^{0}$C. The supernatant was discarded and the pellet was washed by resuspending in 4 ml of 0.0132M phosphate buffer, pH 7.2 (PBSa) and re-centrifuged at 200 x g for 10 min at $4^{0}$C. The washing process was repeated once more to remove debris, if any from the cell lysis, and the pellet was suspended in 500 µl sterile PBSa and was kept on ice until used. The viability of the isolated leukocytes was calculated using 0.1% Nigrosin (Cheesbrough, 2006) within 3 min of isolation. The differential leukocyte counts of isolated leukocytes of each dog were counted on Leishman stained smears to estimate the neutrophil count in each sample to be used in the assay. All samples were analyzed within one and half hours of collection.

### Opsonization of bacterial suspension

A pure culture of *Escherichia coli* (ATCC 25938) in 5 % sheep blood agar (Oxoid, UK) was used in the study. One colony of *E. coli* was inoculated into 5 ml of brain heart infusion broth (Oxoid, UK) and incubated at $37^{0}$C for 18 hrs. The broth culture was centrifuged at 2000 x g for 15 minutes and the resulting pellet was resuspended in 10 ml sterile PBSa.

The optical density of a series of two fold dilutions of the bacterial sample was recorded at 540 nm wavelength using a Jenway 6400 spectrophotometer. The viable bacterial count of each two - fold dilution was determined by the spread plate method (Harrigan & McCance, 1976). Briefly, a tenfold serial dilution was made for each of the above two-fold dilutions using sterile PBSa to calculate the viable bacterial count in each two-fold dilution. The corresponding absorbance for bacterial count of 1 x $10^{6}$/µl was extrapolated from a standard curve plotted with absorbance against $Log_{10}$ bacteria counts. This bacterial concentration was used in all experiments to yield a neutrophil:bacteria concentration of 1:50 for optimum phagocytosis. One hundred microlitre of the above bacterial suspension was mixed separately and individually with 100 µl of serum collected from each animal, and was incubated at $37^{0}$C for 30 min for opsonization. Tubes were gently mixed at 5min intervals. Samples were kept on ice until used for the phagocytosis assay.

### Assay for phagocytic activity

In a single experimental run, 200 l opsonized bacterial suspension was mixed with the calculated volume of neutrophil suspension and incubated at $37^{0}$C. A negative control was made by substituting the bacterial aliquot with PBSa. An aliquot of the incubated sample was used to prepare thin smears for microscopic examination at 15 and 30 min incubation.

### Staining, Microscopic Examination and Statistical Analysis

The above thin air-dried smears of leucocyte-bacteria were stained with Leishman. The percentage of phagocytically active neutrophils (cells with ingested *E. coli*) was determined by counting 5000 neutrophils under oil immersion objective (x1000 magnification) of a light microscope. Paired t-test using Minitab release 14 was used to observe the statistical significance at (P < 0.05), under 95% confidence interval.

### RESULTS AND DISCUSSION

The present study was conducted to investigate the *in vitro* phagocytic activity of blood neutrophils of dogs in Sri Lanka for *Escherichia coli*. The study sample comprised of 70% mongrel and 30% cross bred dogs of both sexes. The leukocyte isolation method described yielded 1,170 - 12,930/il leukocytes of which 61-90% was neutrophils. The viability of the isolated leukocytes ranged from 90-99% (mean 96%). Neutrophils isolated from fresh blood samples of clinically healthy dogs were incubated with *E.coli* which is a facultative gram-negative bacterium found in the normal flora of the gastrointestinal tract. The mean percent phagocytosis (MPP) of canine blood neutrophils for a pure culture of *E. Coli* ATCC 25938, was 70% (63%-73%) and 89 % (78% - 92%) at 15 minute and 30 minute incubation, respectively. Similar phagocytic activity had been recorded for blood neutrophils of cows for *E. coli* which were 74% and 84% at 15 and 30 minutes incubation, respectively (Silva *et al.*, 1989). It was also revealed that MPP of bovine neutrophils was high for *E coli*, Salmonella sp, *Strep. agalactiae* (73-81%); intermediate for *Staph. aureus* (64%), and low for *M. bovis* and *B. abortus* (24 - 40%) (Silva & Jain 1988). The phagocytic activity of buffalo (*Bubalis bubalus*) was slightly different in that the MPP was highest for *Staph aureus* (82%) at 30 min incubation compared to *E. coli*

(77%) and *Strep. agalactiae* (72%) (Silva and Kariyawasam, 1996). When buffalo *(Bubalus Bubalus)* neutrophils were exposed to mixed bacterial cultures, the affinity for phagocytizing *E. coli* was much less in the presence of *Staph. aureus* than in the presence of *Strep. agalactiae* (Silva and Thattil, 1995).

The MPP of neutrophils at 15 min incubation was significantly lower than that at 30 minute incubation (P = 0.00), indicating that 30 minute is required to obtain a better phagocytic activity. It has been shown previously that although phagocytosis was not impaired after 30 or 60 minutes the average bactericidal activity incubation diminishes significantly after 30 minutes (Gosset, 1983). However, the phagocytic activity and postphagocytic bactericidal activity of buffalo neutrophils improved when incubated up to 60 minutes (86% for *E. coli* and 81% for *Strep. agalactiae*) (Silva and Kariyawasam, 1996). Quantitative analysis of phagocytosis and oxidative burst in canine neutrophils performed by flow cytometry techniques showed that storage of canine PMN for 24 hrs at room temperature had no negative influence on phagocytosis or oxidative burst measurements (Eickhoff et al., 2004). Untreated carcinomas and sarcomas in dogs may suppress the oxidative burst of neutrophils while sarcomas may suppress the phagocytic activity of neutrophils (LeBlanc *et al*., 2010). The bactericidal activity during the course of the inflammation did not correlate with neutrophil count, number of toxic neutrophils, or clinical course of the inflammation (Gosset, 1983). These results indicate that a defect in bactericidal activity can occur in dogs with severe inflammatory disease, and that repeated assays, rather than single determinations, may be needed to detect this dysfunction.

A deficiency in immunity lowers the body defenses causing an increased susceptibility to bacterial infections of the skin, respiratory system, and gastrointestinal tract. These infections respond poorly to antibiotics. Immunocompromised animals are less capable of recovering from infections due to an improperly functioning immune response. Though congenital or inherited immunodeficiency disorders are uncommon in the dog, immunodeficiency secondary to a wide range of causes (age, drug therapy, and chronic neoplastic or infectious disease) is relatively common in adult dogs. Canine leukocyte adhesion deficiency (CLAD) is a primary immunodeficiency disease in Irish Setter Dogs characterized by recurrent bacterial infections in the presence of marked leukocytosis (Foureman *et a*l., 2002) caused by a mutation in the beta-2 integrin subunit gene (ITGB2). The causative mutation results in an amino acid change from cysteine to serine at amino acid 36 (Cys36Ser) in the beta-2 integrin subunit protein. Despite massive neutrophilia, these cells cannot egress into tissues in a range of infectious, inflammatory allergic and auto immune diseases. Phagocytosis is an essential mechanism of the innate and adaptive immune system. A deficiency in phagocytosis can be due to a low number of phagocytes

in the blood, viral infection, such as Distemper, or a congenital defect.

Products of bacteria activate the expression of glycoprotein on capillary endothelial surface, tether neutrophils and stimulating them to roll along the endothelium (Tizard, 2009; Kaneko, 1997). The platelet activating factor (PAF) secreted by the endothelial cells, activate the rolling neutrophils to express the adhesive protein integrin, which allows the neutrophils to bind strongly to a glycoprotein intercellular adhesion molecule-1 (ICAM-1; CD54) expressed on the endothelial cells. This strong binding allows the rolling neutrophils to attach firmly to the vessel wall despite the shearing force of the blood flow. Younger dogs have significantly higher levels of mRNA for interleukin-8 receptor (IL-8R), l-selectin and interleukin-1beta-converting enzyme (ICE), specific for neutrophil migration or killing functions. A significant effect of age on percent bacterial killing by neutrophils has been shown in Beagle dogs, with a decline in phagocytic ability from 39% to 25% at 4 and 10 years of age, respectively, suggesting that older dogs have depressed innate immune responses compared with younger dogs (Hall *et al*., 2010).

The findings support the fact that the innate immunity of dogs can be measured qualitatively and quantitatively and the technique described can be useful for the identification of immunocompromised dogs.

## REFERENCES

Abbas, A., Lichtman, A.H., Pillai, S. (2007). Innate Immunity. In: Cellular and Molecular Immunology, 6th Edition: Saunders.

Cheesbrough, M., (2006). Laboratory practice in tropical countries part 01: pp.406. Cambridge University Press.

Eickhoff, S., Mironowa, L., Carlson, R., Leibold, W., Tipold, A. (2004). Measurement of phagocytosis and oxidative burst of canine neutrophils: high variation in healthy dogs. *Veterinary Immunology and Immunopathology*, **101**(1-2**)**:109-21.

Foureman P, Whiteley M, Giger U. (2002). Canine leukocyte adhesion deficiency: presence of the Cys36Ser beta-2 integrin mutation in an affected US Irish Setter cross-breed dog and in US Irish Red and White Setters. *Journal of Veterinary Internal Medicine*, **16(5)**:518-23.

Gershwin, L.J., Krakowka, S., Olsen, R.G., (1995). Assays for innate immune defense. Immunology and immunopathology of domestic animals 2nd Edition:71-75. Mosby-Year Book, Inc., St Louis, USA.

Gosset, K.A., Mac Williams, P.S., Enright, F.M., Cleghorn, B. (1983). In vitro function of canine neutrophils during experimental inflammatory disease. *Veterinary Immunology and Immunopathology*, **5**: 151-159

Hall, J.A., Chinn, R.M., Vorachek, W.R., Gorman,

M.E., Jewell DE. (2010). Aged Beagle dogs have decreased neutrophil phagocytosis and neutrophil-related gene expression compared to younger dogs. Veterinary Immunology and Immunopathology, **137(1-2)**:130-5.

Harrigan, W.F., Mccanae, M.E., (1976) Laboratory method in food and dairy microbiology. Academic press, lnc: Limited, London

Jain, N.C. (1986). Schalm's veterinary hematology, **4**[th] Edition: Lea & Febiger, Philadelphia, USA.

Kaneko, J, Harvey, J, Bruss, M (1997). Clinical Biochemistry of Domestic Animals. eBook ISBN: 9780080529196, Academic Press:285-289.

LeBlanc, C.J. LeBlanc, A.K., Jones, M.M., Bartges, J.W., Kania, S.A. (2010). Evaluation of peripheral blood neutrophil function in tumor-bearing dogs. *Veterinary Clinical Pathology*, **39**(2): 157163

Opal, S.M., Cross, A.S. (2005). The use of immunocompromised animals as models for human septic shock. Shock, **24** (Suppl 1):64-70.

Roitt, I. M., (1988 ).The basis of immunology. Essential immunology.**6**:1-13

Silva, I.D. and Mallawa, M.R.C.K. (2010). A monograph on clinical hematology and veterinary diagnostic techniques, Department of Veterinary Clinical Sciences, Sri Lanka.

Silva I.D., Kariyawasam, S. (1996). Postphagocytic bactericidal activity of buffalo (*Bubalis Bubalus*) neutrophils against mastitis causing bacteria. *Journal of the National Science Council of Sri Lanka*, **24**(2): 71-80.

Silva I.D., Thattil, R.O. (1995). Preferential phagocytosis of bacteria in mixed cultures by buffalo (*Bubalis Bubalus*) neutrophils. *Journal of the National Science Council of Sri Lanka*, **23**(1): 9-15.

Silva, I. D., Jain, N. C., George L.W. (1989). Phagocytic and nitroblue tetrazolium reductive properties of mature and immature neutrophils and eosinophils from blood and bone marrow from cows. *American Journal of Veterinary Research*, **50** (5)**:** 778-781.

Silva, I.D., Jain, N.C., (1988). Phagocytic and nitroblue tetrasolium reductive properties of bovine neutrophils for mammary phathogen. *Journal of dairy science,* **71**:1625-1631

Silva, I. D., Jain, N. C., Farver, T. B., Zinkl, J. G., (1988). Phagocytic and Post phagocytic activities of bovine neutrophils for pure and mix cultures. *Journal of dairy science*, **71**:2513-2519.

Tizard, I.R. (2009). Neutrophils and their products. Veterinary Immunology and introduction. 8th Edition: CBS Publishers & Distributors: 21-43.