UNIVERSITI PUTRA MALAYSIA

IMMUNOLOGICAL RESPONSE OF SHEEP TO *EPERYTHROZOON OVIS* INFECTION

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By

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Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Veterinary Science

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DEDICATION

To my parents, family members and beloved wife for their kind support all these while and to the Lotus Feet of Lord Sri Balasubramaniyar, Taman Seri Timah, Balakong

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree Masters in Veterinary Science

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The immunity of sheep to *Eperythrozoon ovis (E.ovis)* has been investigated through the peripheral blood smears stained with Giemsa. A naturally infected flock monitored for a year revealed the activity of peripheral blood monocytes to be involved in active phagocytosis of infected erythrocytes; a process called erythrophagocytosis. Although neutrophils, lymphocytes and thrombocytes were found to be activated in the initial stage of immune response, the monocytes seemed to predominate the phagocytosis at the later stage of infection during erythrophagocytosis. At all stages and degree of infections, no obvious anaemia, jaundice and emaciation were observed in these well fed sheep flocks. Anaemia was observed in flocks where malnourishment and stress conditions were present with a consistent high degree of parasitaemia. *E.ovis* infection trial in mice

exhibited more lymphocytic activities compared to the sheep , although lymphocytes, neutrophils and thrombocytes were involved in the early enhancement of inflammatory process against *E.ovis* as per in the sheep. These inflammatory processes were observed at day 20 post infection in mice. Similarly, only monocytes were found to be actively involved in erythrophagocytosis at the later stage of infection prior to the disappearance of the organisms from the peripheral circulation. Increased Kupffer cell activity showed liver was also involved in the removal of infected erythrocytes besides the blood peripheral macrophages.

In vitro phagocytosis assay using the Acridine Orange as the flurochrome revealed that peripheral monocytes ingested around eight cells of *E.ovis* per monocyte within 30 minutes upon contact. These cells were also killed within 30 minutes upon ingestion, characterised as red cells within the cytoplasm of monocytes. The Enzyme–linked Immunosorbent Assay was possible for optimization and was not suitable for further development as the Lang's method yields impure antigen from blood lysates. Latex test development was hindered due to the various host and immune serum factors that have resulted in non- specific agglutinations.

The persistence of infection in the flock throughout the one - year period of observation signified that sheep had been constantly infected with *E.ovis* and remained carriers for a very long period. The persistent parasitemia may suggest that the immunity to the parasite has been very complex probably due to highly

diversed antigenic variants, a characteristic exhibited by most rickettsiae in the Order of *Rickettsiales* or as a result of detrimental effects of the organism on the immune mechanism.

Sheep flocks naturally infected with *E.ovis* have remained permanent carriers. The findings from this research suggest that the sheep was unable to confer an effective or protective immune response against the pathogen. Peripheral blood macrophages are the most important first line of defense in removing the *E.ovis* from the peripheral blood.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains Veterinar

TINDAKBALAS IMUN BEBIRI TERHADAP JANGKITAN *EPERYTHROZOON OVIS*

Oleh

SHANKAR GANESH KANABATHY

Jun 2004

Pengerusi : Profesor Madya Che Teh Fatimah Nachiar Iskandar, Ph.D.

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Tindakbalas imun bebiri terhadap jangkitan *Eperythrozoon ovis (E.ovis)* telah dikaji melalui saringan darah yang diwarnakan dengan pewarna Giemsa. Kumpulan bebiri yang telah dijangkiti secara semulajadi telah disaring selama setahun. Ianya menunjukan bahawa monosit darah memainkan peranan yang penting dalam membunuh sel darah merah yang telah dijangkiti *E.ovis,* proses yang dikenali sebagai 'erythrophagocytosis'. Walaupun neutrofil, limfosit dan trombosit dirangsang pada peringkat permulaan inflamasi; monosit merupakan sel yang terus menjalankan proses 'erythrophagocytosis' sehingga akhir proses ini. Pada semua peringkat inflamasi, tiada tanda-tanda kurang darah diperhatikan pada kumpulan bebiri ini. Kekurangan darah telah diperhatikan pada kumpulan bebiri yang tidak mempunyai sumber makanan yang mencukupi. Ujikaji jangkitan *E.*

ovis dalam tikus menunjukkan aktiviti limfosit yang lebih ketara berbanding bebiri walaupun neutrofil dan trombosit turut serta dalam proses inflamasi terhadap *E.ovis.* Proses inflamasi ini diperhatikan berlaku dalam jangkamasa 20 hari dari tempoh jangkitan tikus. Seperti dalam bebiri, monsit merupakan sel yang aktif dalam proses 'erythrophagocytosis' atau proses pemakanan sel darah merah. Pertambahan aktiviti sel Kupffer di dalam hati menujukkan bahawa hati turut serta dalam proses pemusnahan dan penyingkiran sel darah merah yang dijangkiti *E.ovis*.

Ujikaji pemakanan *in vitro E .ovis* oleh monosit telah dijalankan dengan menggunakan 'Acridine Orange' sebagai fluorokrom. Eksperimen ini menunjukan bahawa setiap monosit memakan hampir 8 sel *E .ovis* dalam masa 30 minit dari masa pertemuan. Sel *E. ovis* telah dibunuh sepenuhnya dalam masa 30 minit ini, diperhatikan sebagai sel yang telah menjadi merah sepenuhnya dalam sitoplasma monosit. Penghasilan sistem 'Enzyme-linked Immunosobent Assay' berjaya ke tahap penentuan faktor-faktor optima sahaja disebabkan kewujudan reaksi tak spesifik berpunca dari antigen yang tak tulin melalui kaedah Lang. Penghasilan 'Latex Test' juga tergendala disebabkan reaksi tak spesifik berpunca dari faktor haiwan dan sera bahan ujikaji .

Jangkitan *E. ovis* yang berterusan pada kumpulan bebiri selama setahun menunjukan bahawa bebiri sentiasa dijangkiti oleh *E. ovis* dan status pembawa telah wujud untuk masa yang berpanjangan. Jangkitan terus menerus ini

menunjukan bahawa tahap imun kepada parasit ini amat kompleks, mungkin disebabkan ciri-ciri organisma ini yang luas variannya; ciri-ciri yang biasa ditunjukan oleh patogen – patogen dalam Order '*Rickettsiales*'; atau disebabkan oleh kesan buruk patogen ini terhadap sistem imun haiwan.

Kumpulan bebiri yang dijangkiti *E.ovis* secara semulajadi menjadi pembawa penyakit ini. Hasil kajian ini menunjukkan bahawa bebiri gagal mempertahankan dirinya dari segi tindakbalas imuniti terhadap patogen ini. Makrofaj darah merupakan pertahanan badan yang pertama dalam pemusnahan *E.ovis* dalam darah.

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Veterinary Science. The members of the Supervisory Committee are as follow :

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

SHANKAR GANESH KANABATHY

Date :

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LIST OF ABBREVIATIONS

AO	Acridine Orange
BSA	Bovine Serum Albumin
CFT	Complement Fixation Test
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
g	Gram
Hb	Hemoglobin
HBSS	Hank's Balanced Salt Solution
IFAT	Immunofluorescent Antibody Test
IFN-γ	Interferon-gamma
IHA	Indirect Hemagglutination Test
LAT	Latex Agglutination Test
mg	Milligram
ml	Millilitre
NK	Natural Killer
OD	Optical Density
PBS	Phosphate Buffered Saline
PVP	Polyvinylpyrollidone
PBST	Phosphate Buffer Saline Tween
RBC	Red Blood Cell
SPF	Specific Pathogen Free
TMB	Tetramethylbenzidine
µl	Microlitre

CHAPTER 1

INTRODUCTION

The economic losses due to *Eperythrozoon ovis (E.ovis)* infection in the sheep industry are reduced wool and reproduction, poor wool growth (Kreier and Ristic, 1963), increased risk to other diseases in chronically infected animals and mortality (Gulland *et al*, 1987). *Eperythrozoon ovis* has been isolated from sheep in many countries and usually produces mild clinical signs in experimentally inoculated animals. In some circumstances it is associated with a severe disease in young sheep known as ill-thrift. Ill-thrift is restricted to certain geographic situation and has been reported from Australia, New Zealand, France, Norway and South Africa, characterized by a failure of young sheep to thrive when sheep of all other ages appear to be in good health and weight gain (Stewart, 1981). The first report on eperythozoonosis in Malaysia was by Fatimah *et al*, (1994) from a sheep concurrently suffering from copper toxicity. Mariah *et al*, (1997) reported that the morphology of *E. ovis* in sheep and goats as being coccoid and rod - like in sheep.

The organism is classified based on the species of animals being infected: *Eperythrozoon wenyoni* in cattle, *Eperythrozoon suis* in pigs and *Eperythrozoon ovis* in sheep and goat. The identification of the organism is based on the demonstration of antigen and the antibodies. Prior to 1970, the diagnosis of eperythrozoonosis was based on herd and individual animal histories of

icteroanemia and demonstration of eperythrozoon bodies in blood smears stained by Giemsa. In ovine and bovine eperythrozoonosis, complement fixation test (CFT), enzyme linked immunosorbent assay (ELISA) and passive hemagglutination tests have been developed to detect micro-organisms in the circulating red blood cells (Daddow , 1977; Finerty *et al* ,1969; Kawazu *et al* , 1990; Lang *et al* , 1987). Smith and Rahn (1975) developed an indirect hemagglutination test (IHA) for measuring antibodies to *E.suis*. Smith (1981) reported that IHA negative pigs could be carriers because parasitemia was observed in IHA negative pigs after splenectomy, indicating that the test may lack sensitivity for detecting chronically infected carriers.

The diagnosis of *E.ovis,* based mainly on peripheral blood smear can sometimes be difficult because this test may not be specific and is done on unclotted red blood cells which are frequently lysed in transit. A modified indirect immunofluorescent antibody test (IFAT) was used for easy and specific diagnosis of *E.ovis* from field samples (Ilemobade and Blotkamp, 1978). Antibody levels in the complement fixation test disappeared by 80 days on average and that the test is of limited value in detecting previous infection or exposure (Daddow, 1977). The coating antigen used in ELISA was a crude preparation from infected red blood cell lysates and contained host red blood cell antigens. The presence of host proteins in ELISA coating antigens may interfere with the test results (Hsu *et al*, 1992; Schuller *et al*, 1990). In general, ELISA positive results are significant, but negative results provide no information on the infection status of the animal.

Serological tests for the diagnosis of eperythrozoon have limitation due to marked variability in antibody response, as well as a failure to identify acutely infected sheep. Due to the lack of an efficient test to identify sheep and goats which are chronically or latently infected with *E.ovis*, the true incidence and economic impact of subclinical *E.ovis* infection is unknown.

Molecular techniques such as polymerase chain reaction (PCR), deoxyribonucleic acid (DNA) sequencing and Western blotting offer virtually unlimited opportunities to improve the ability to study and diagnose disease. As diagnostic tools, they have a better sensitivity and specificity compared to most immunological tests (Cox *et al*, 1991; Deacon and Lah, 1989; Peter, 1991). These techniques also offer a means of studying the genetic and pathologic basis of disease at the molecular level.

E.ovis has been detected in Malaysia back in 1980's and lately by Fatimah *et al*, (1994) from a sheep suffering from copper toxicity. Losses due to ovine eperythrozoonosis in Malaysia is unknown and it is therefore difficult to determine losses in an individual flock because of the lack of complete understanding of the nature of the disease and definitive diagnostic aids. However, a diagnostic tool such as the ELISA to detect the antibodies to *E.ovis* is sufficient to enable us to understand the prevalence rate of previous and current exposure. Besides, ELISA too allows the screening of a large number of samples in a day.

The report on erythrophagocytosis in peripheral blood, to date, is yet to be reported and there have been great discrepancies in reports on the role of humoral antibody in protecting sheep from reinfection. As parasitaemia fluctuates in the peripheral blood, a rapid field diagnosis is required for identification of infected animals . Therefore, the hypotheses and objectives of the study were as follow :

The hypotheses were :

(1) A high antibody titre is correlated with a low level of parasitemia due to *E.ovis*.

(2) Macrophage plays an important role in phagocytosis and elimination of *E.ovis* in peripheral blood.

The objectives of this study were:

(1) To demonstrate the role of macrophages in phagocytosing E.ovis and elimination of the organisms from the peripheral blood.

(2) To develop a Latex Test as an alternative for rapid diagnosis of *E.ovis* in the field

(3) To determine the antibody level at different phases of parasitemia using ELISA.