EFFECT OF SINGLE INFECTION OF *PLASMODIUM BERGHEI* AND COINFECTION WITH *BRUGIA PAHANGI* IN GERBILS

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EFFECT OF SINGLE INFECTION OF *PLASMODIUM BERGHEI* AND COINFECTION WITH *BRUGIA PAHANGI* IN GERBILS

ABSTRACT

Malaria and lymphatic filariasis (LF) are two leading and common mosquito-borne parasitic diseases worldwide. These two diseases are co-endemic in many tropical and sub-tropical regions and are known to share vectors. The interactions between malaria and filaria parasites are poorly understood. Thus, this study aimed at establishing *Plasmodium berghei* ANKA (PbA) infection in gerbils and understanding its interactions with *Brugia pahangi* co-infections. Briefly, the gerbils were matched according to age, sex and weight and grouped into filaria-only infection, PbA-only infection, co-infection and control group. Filaria infection was established by inoculating 50 infective larvae of *B. pahangi* subcutaneously into each experimental gerbil. After a prepatent period of 70 days of *B. pahangi* infection, co-infection was initiated with the inoculation of 10^6 PbA infected red blood cells (iRBCs) intraperitoneally, whereas the PbA-only infected group of gerbils were initially given 0.2 mL phosphate buffered salt subcutaneously before being given 10^6 PbA iRBCs intraperitoneally after 70 days. The parasitaemia, survival and clinical effect on the gerbils were monitored for a period of 30 days post *Plasmodium* infection. The immune responses of gerbils to both mono and co-infection were monitored together with assessment of histopathology of the infections. Findings show that gerbils were susceptible to PbA infection, showing significant decrease in haemoglobin concentration, RBC counts, body weight and temperature, over the course of infection. However, there were no neurological signs observed and the death of gerbils might be as a result of severe anaemia. Results on co-infection experiments showed that co-infected gerbils survived longer than PbA-infected gerbils. Food and water consumption were significantly reduced in both PbA-infected and co-infected gerbils, although loss of body weight, hypothermia, splenomegaly and hepatomegaly were less

severe in co-infected gerbils. *Plasmodium* infected gerbils also suffered hypoglycemia which was not observed in co-infected gerbils. Histopathology reveals that the presence of *B. pahangi* adults and microfilariae in the lungs of gerbil, invoked inflammatory responses and damages to the lungs in both filaria-only and co-infected gerbils. Furthermore, gerbil cytokine responses to co-infection were significantly higher than PbA-only infected gerbils, which is being suggested as a factor for their increase survivability. Co-infected gerbils had significantly elicited interleukin-4, interferon-gamma and tumour necrotic factor at early stage of infection than PbA infected gerbils. Findings from this study suggested that *B. pahangi* infection protected against severe anaemia, hypoglycemia, haemorrhagic alveolitis and bronchitis which are manifestations of PbA infection. Therefore, filaria infection seems to protect against severity of PbA infection in gerbils.

Keywords: Gerbil, *Plasmodium berghei* ANKA, *Brugia pahangi*, Co-infection.

KESAN MENGGUNAKAN SINGAPURA *PLASMODIUM BERGHEI* DAN KESALAHAN DENGAN KOINFEKSI *BRUGIA PAHANGI* DI GERBILS

ABSTRAK

Malaria dan filariasis limfatik adalah dua penyakit parasit utama dan penyakit bawaan-nyamuk yang lazim diseluruh dunia. Kedua-dua penyakit ini adalah koendemik dibanyak kawasan tropika dan subtropika dan diketahui berkongsi vektor. Interaksi diantara parasit malaria dan filaria tidak diketahui dengan baik. Oleh itu, kajian ini bertujuan untuk membuktikan jangkitan *Plasmodium berghei* ANKA (PbA) dalam gerbil dan memahami interaksi ko-infeksi dengan *Brugia pahangi*. Secara ringkas, gerbil dipadankan mengikut umur, jantina dan berat badan dan dikumpulkan dalam kumpulan hanya jangkitan filaria, hanya jangkitan PbA, ko-infeksi dan kumpulan kawalan. Jangkitan filaria dilakukan dengan menginokulasi 50 larva infektif *B. pahangi* secara subkutaneus ke dalam setiap gerbil ujikaji. Selepas jangkamasa prepaten 70 hari bagi jangkitan *B. pahangi*, ko-infeksi telah dimulakan dengan menginokulasi 10^6 sel darah merah yang terjangkit PbA secara intraperitoneal dan sebaliknya kumpulan gerbil terjangkit dengan hanya PbA telah diberi 0.2 mL garam phosphate buffer secara subkutaneus pada permulaannya sebelum diberi 10^6 darah merah yang terjangkit PbA secara intraperitoneal selepas 70 hari. Parasitemia, daya hidup dan penilaian klinikal bagi gerbil telah dipantau untuk jangkamasa 30 hari selepas jangkitan *Plasmodium*. Tindak balas imun bagi gerbil pada kedua-dua mono dan ko-infeksi telah dipantau, bersama-sama dengan penilaian histopatologi bagi jangkitan itu. Penemuan menunjukkan yang gerbil mudah terkena jangkitan PbA dan menunjukkan penurunan signifikant pemekatan hemoglobin, bilangan sel darah merah, berat badan dan suhu, semasa jangka waktu jangkitan. Bagaimanapun, tidak ada tanda neurologi yang dilihat dan kematian gerbil mungkin disebabkan oleh anemia yang teruk. Keputusan bagi ko-infeksi menunjukkan, gerbil yang telah diko-infeksi mempunyai kadar daya hidup yang lebih tinggi dari gerbil yang terjangkit dengan PbA. Pengambilan makanan dan air

telah berkurang secara signifikant dalam kedua-dua PbA terjangkit dan gerbil yang telah mengalami ko-infeksi, walaupun kehilangan berat badan, hipotermia, anemia, splenomegali dan hepatomegali adalah kurang teruk dalam gerbil yang mengalami ko-infeksi. Gerbil yang dijangkiti *Plasmodium* juga menderita hipoglisemia yang tidak dilihat dalam gerbil yang mendapat ko-infeksi. Histopatologi menunjukkan yang kewujudan cacing dewasa dan mikrofilaria *B. pahangi* dalam paru-paru gerbil, mencetuskan tindak balas inflamatori dan kerosakan pada paru-paru dalam kedua-dua gerbil yang dijangkiti oleh hanya filaria dan gerbil yang mengalami ko-infeksi. Selain itu, tindak balas sitokin gerbil dalam ko-infeksi adalah lebih signifikant dari gerbil yang hanya dijangkiti oleh PbA, dan dicadangkan sebagai faktor bagi peningkatan daya hidup. Gerbil yang mengalami ko-infeksi menerbitkan interleukin-4, interferon-gamma dan faktor nekrotik tumor pada peringkat awal jangkitan secara signifikant dari gerbil yang terjangkit dengan PbA. Penemuan dari kajian ini mencadangkan yang jangkitan *B. pahangi* melindungi daripada anemia teruk, hipoglisemia, alveolitis dan bronkitis hemorhagik yang merupakan manifestasi jangkitan PbA. Oleh itu, jangkitan filaria seolah-olah melindungi daripada jangkitan PbA yang teruk dalam gerbil.

Katakunci: Gerbil, *Plasmodium berghei* ANKA, *Brugia pahangi*, ko-infeksi.

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LIST OF SYMBOLS AND ABBREVIATIONS

Ab	:	Antibody
AIDS	:	Acquired immune deficiency syndrome
AIM	:	Action and investment to defeat malaria
AMA	:	Apical membrane antigen 1
ARD	:	Acute respiratory distress syndrome
BLAST	:	Basic local alignment search tool
cDNA	:	Complementary deoxyribonucleic acid
CM	:	Cerebral malaria
DC	:	Dendritic cell
DEPC	:	Diethylprocarbonate
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxynucleotide
DPX	:	Distyrene plasticizer and xylene
DSP	:	Diurnal sub periodic
DTT	:	Dithiothreitol
EBA175	:	Erythrocyte binding antigen-175
FFPE	:	Formalin fixed paraffin embedded
GAPDH	:	Glyceraldehyde-3-phosphate dehydrogenase
GPELF	:	Global programme to eliminate lymphatic filariasis
GTS	:	Global technical strategy
H & E	:	Haematoxylin and eosin
Hb	:	Haemoglobin
HRP2	:	Histidine rich protein 2
HRP3	:	Histidine rich protein 3

ICT	:	Immuno-chromatographic test
IFN-γ	:	Interferon gamma
IgG	:	Immunoglobulin G
IL-10	:	Interleukin 10
IL-4	:	Interleukin 4
IL-6	:	Interleukin 6
iRBCs	:	Infected red blood cells
IRS	:	Indoor residual spraying
ITN	:	Insecticide-treated mosquito nets
KC	:	Kupffer cells
LF	:	Lymphatic filariasis
MCP-1	:	Monocyte chemoattractant protein 1
MDA	:	Mass drug administration
mRNA	:	Messenger Ribonucleic acid
MSP-1	:	Merozoite surface protein 1
NK	:	Natural killer
NKT	:	Natural killer T-cells
NP	:	Nocturnal periodic
NSP	:	Nocturnal sub periodic
PAM	:	Placental associated malaria
PbA	:	*Plasmodium berghei* ANKA
PBMCs	:	Peripheral blood mononuclear cells
PBS	:	Phosphate buffered salt
pi	:	Post infection
RBCs	:	Red blood cells
RDT	:	Rapid diagnostic test

RNA	:	Ribonucleic acid
RT	:	Room temperature
SDGs	:	Sustainable development goals
SEM	:	Standard error of mean
SMA	:	Severe malaria anaemia
SSC	:	Standard sodium citrate
TGF	:	Tumour growth factor
TNF	:	Tumour necrotic factor
WHO	:	World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 GENERAL INTRODUCTION

Malaria and lymphatic filariasis (LF) are the two most important mosquito-borne parasitic diseases responsible for high morbidity worldwide. Lymphatic filariasis is often found to be endemic in the same region or country where malaria is also endemic (Figure 1.1) hence, signifies the presence of co-infection in humans. Both diseases have been identified to share common vectors (Manguin et al., 2010). Malaria is an acute form of a disease which can be asymptomatic, uncomplicated or severe, whereas LF is a chronic disease which can either lead to mild pathology with high parasite burden or severe pathology such as lymphatic inflammation and elephantiasis (McSorley & Maizels, 2012), and the host can harbour the parasite for decades.

As of 2015, about half of the world population was at risk of having malaria, with 214 million cases and 438 thousand deaths were recorded (WHO, 2016a). About 947 million people living in 54 countries are at risk of lymphatic filariasis, while an estimated 25 million men still suffer genital disease and over 15 million people are afflicted with lymphoedema (WHO, 2016b).

Adults in malaria endemic areas are usually asymptomatic (though harbouring the parasites) due to their acquired semi-immunity over time, whereas it is contrary in low transmission areas (Langhorne et al., 2008). The clinical manifestations of malaria are mainly determined by multiple immunological responses and physiological balances (Deroost et al., 2016). The parasite metabolic or secretory products such as heme, hemozoin, and the parasite DNA often provoke the host immune response in order to stop the parasite multiplication (Deroost et al., 2016). This response is often initiated by the innate immune activities of natural killer cells and phagocytic cells. At early stage of the erythrocytic infection, pro-inflammatory cytokines are highly elevated (Artavanis-

Tsakonas & Riley, 2002; Good et al., 2005). Conversely, at the later stage of malaria infection CD4+ T cells modulate the Th1 cells to a Th2 profile of cytokines, which aid the B cells in killing the parasites (Hartgers & Yazdanbakhsh, 2006; Langhorne et al., 1998). However, balance between the pro-inflammatory and anti-inflammatory responses is very important for the host to avoid immunopathology (Deroost et al., 2016).

On the other hand, immune response to helminths has shown to be polarized towards Th2 cytokines such as interleukin-4 (IL-4), IL- 5 and IL-13. In spite of the strong type 2 responses elicited by the helminths, adult worms survive for many years in the vertebrate host, which has been attributed to the ability of the helminth worms to modulate host immune responses and hence become tolerant to the host (Hartgers & Yazdanbakhsh, 2006). The mechanisms underlying the Th2 dominant immune response against helminths is being initiated by modulation of the innate immune system, which in turn gives rise to the anti-inflammatory environment (Hartgers & Yazdanbakhsh, 2006). The immunomodulation is being frontiered by immunomodulatory cytokines, IL-10 and tumour growth factor beta (TGF-β). Hence, the hypo-responsiveness of the host to helminths infection can be suggested to be favorable to both parasite and host, whereby the parasite is being prevented from elimination and continues to grow and multiply, while concurrently the host is being prevented from excessive inflammation which could result in pathology (Maizels & Yazdanbakhsh, 2003; Taylor et al., 2005).

Reports from co-infection of helminths and malaria in humans have shown two possible clinical outcomes; increase in malaria incidence and pathology among helminth infected individuals, and helminth infections protecting against severity of malaria infection (Dolo et al., 2012; Metenou et al., 2009; Nacher, 2008). There are 2 hypotheses that have been suggested as to why helminths might protect malaria parasite from host immune rigor. The first is weakening of the proinflammatory responses through mutual

inhibition of the Th1 response towards the malaria parasite, at the expense of the Th2 response towards the helminth worms (Hartgers & Yazdanbakhsh, 2006; Jankovic & Sher, 2001; Specht & Hoerauf, 2007). Secondly, the regulatory T cells provoked by the helminth worms often aid in the suppression of all types of cellular effector mechanisms (Maizels & Yazdanbakhsh, 2003; Wammes et al., 2010). Nevertheless, the same hypotheses have also been analyzed to suggest that the modulation of the proinflammatory cytokines could actually favour the growth and multiplication of the malaria parasite and hence, aggravate the severity of malaria infections (Le Hesran et al., 2004; Nacher et al., 2002; Spiegel et al., 2003).

Furthermore, studies on animal models of filaria co-infection with rodent malaria parasites are lacking. Among the few studies, the filaria nematode, *Litmosoides sigmodontis* has been used extensively in combination with different malaria parasites such as *Plasmodium yoelii*, *P. chabaudi* and *P. berghei* ANKA, on different mouse strains, notably BALB/c and C57BL/6 (Karadjian et al., 2014; Specht et al., 2010). Moreover, it is only Yan et al. (1997) that have shown the use of attenuated larvae of *Brugia pahangi* co-infected with *P. berghei* in CBA/J mice. The less frequent use of *Brugia* species in co-infection models can be attributed to non-availability of suitable murine host other than gerbils. It is noteworthy that gerbils have rarely been used as experimental model for malaria. Hence, the present study explores new animal model for *B. pahangi* co-infection with *P. berghei* ANKA in gerbil.

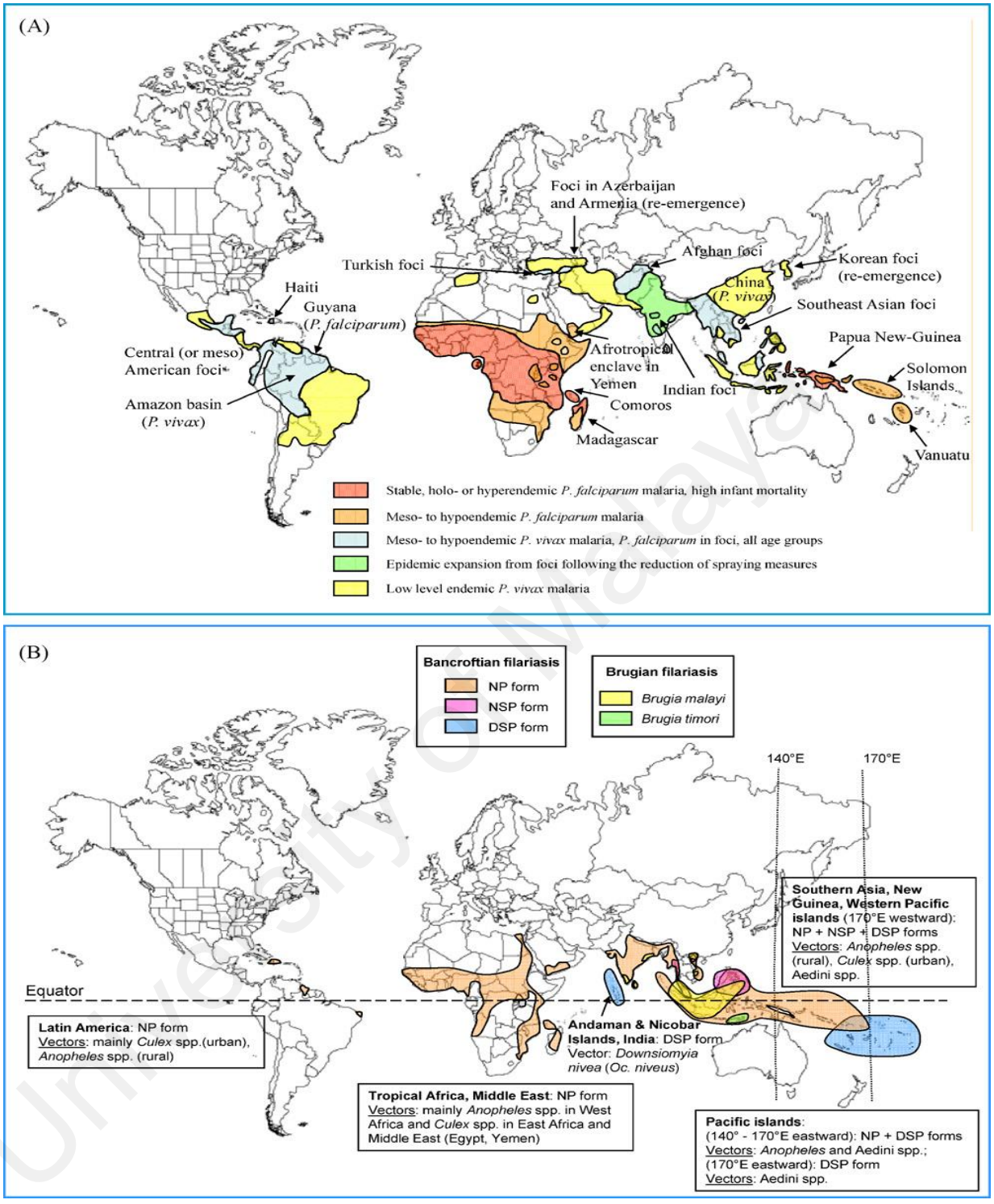


Figure 1.1: Global distribution of malaria and lymphatic filariasis.

A. Malaria, B. Lymphatic filariasis. Adapted from (Manguin et al., 2010). NP: nocturnal periodic; NSP: nocturnal sub-periodic; and DSP: diurnal sub-periodic.

1.2 STATEMENT OF RESEARCH PROBLEM

Co-infection of a host by multiple parasite species has important epidemiological and clinical implications. The magnitude or interactions of these multiple or co-infections vary considerably among systems. However, it has been hypothesized that filaria nematodes polarize immune system toward T helper cells 2 (Th2), thereby increasing the survival and multiplication of malaria parasites, and thus exacerbate malaria outcome; and the modulation of immune responses by filaria nematodes ameliorates the severity of malaria pathology. The debate is still hanging on the outcomes of co-infection which still boarders on different parasites and hosts combinations, resulting in varying and contradicting outcomes. Hence, there is no general frame work to understudy the mechanisms on the outcomes of co-infection of malaria and helminths in experimental models.

Thus, the present study investigated the interactions between *B. pahangi* and *P. berghei* ANKA co-infection in an experimental animal model, gerbil (*Meriones unguiculatus*).

1.3 OBJECTIVES OF STUDY

1.3.1 Main objective

The main objective of this study is to establish an animal model for co-infection of *B. pahangi* and *P. berghei* ANKA, and to investigate the effect of co-infection between both parasites in the same host.

1.3.2 Specific objectives

- To establish the susceptibility of gerbil to *P. berghei* ANKA infection.
- To determine the susceptibility of patent filaria infection in gerbil to *P. berghei* ANKA infection.

- To characterise the effects of patent filaria infection on *P. berghei* ANKA infection.
- To assess the immune response profile of gerbil to co-infection.
- To examine the pathological effects of co-infection on the gerbil host.

1.4 HYPOTHESIS

- *P. berghei* ANKA infection in gerbil serves as a good model for severe malaria.
- Patent filaria infection in gerbil protects against severity of *P. berghei* ANKA infection by elongating the lifespan of co-infected gerbils and protect against severe malaria.
- Patent filaria infection in gerbil induces earlier proinflammatory and anti-inflammatory immune response against *P. berghei* ANKA infection.

1.5 BENEFIT OF STUDY

The present study aims to establish gerbil as an experimental model for severe malaria and also, co-infection model for *B. pahangi* and *P. berghei* ANKA. The present study contributes further to the existing knowledge on the interactions that interplay between *B. pahangi* and *P. berghei* ANKA in co-infection, by showcasing the influence of *P. berghei* ANKA infection in dictating the disease manifestations and the filaria parasite protect the host to some level against the severity of malaria infection. The outcome of this study calls for caution in the control and elimination of lymphatic filariasis, against the possibility of negative effect on transmission of malaria in the endemic areas.

CHAPTER 2: LITERATURE REVIEW

2.1 MALARIA

2.1.1 Malaria infection

Malaria is one of the leading life-threatening diseases in the tropics, where it causes death and morbidity in endemic regions. In the year 2015, about 88% of deaths due to malaria were recorded in WHO Africa region, while 10% of malaria deaths were observed in WHO Southeast Asia region and WHO Eastern Mediterranean region accounted for the remaining 2% (WHO, 2016a). The disease is caused by a protozoan parasite from the genus *Plasmodium*, which is transmitted through infected female *Anopheles* mosquitoes.

There are about 400 known species of *Anopheles* mosquitoes, among which approximately 70 species are of epidemiological importance (Manguin & Boëte, 2011). The biodiversity of Anopheline has influence on the epidemiology of malaria. The composition of *Anopheles* mosquitoes in an area or community is greatly affected by factors such as demographic, ecological and climate (temperature, humidity and rainfall) changes (Manguin & Boëte, 2011; Manguin et al., 2008), and hence, has significant impact on the transmission of malaria.

2.1.2 Malaria parasites

Malaria is caused by the parasite *Plasmodium*, which belongs to the genus of parasitic protists. *Plasmodium* belongs to the family Plasmodiidae, order Haemosporida and phylum Apicomplexa. There are over a hundred *Plasmodium* species, which infect humans and animals such as primates, rodents, reptiles and birds. There are 5 species of *Plasmodium* that cause malaria in humans, *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (Figtree, 2010; Singh et al., 2004; Vythilingam et al., 2006; White, 2008). Until recently, *P. knowlesi* has been known to cause simian malaria in macaques (Sinton & Mulligan, 1933).

Plasmodium falciparum is known to be the most virulent and pathogenic, among the human malaria parasites. *Plasmodium falciparum* can be found in the tropics and sub-tropical areas and is the most prevalent on the continent of Africa. It is also responsible for most deaths from malaria (WHO, 2016a). On the other hand, *P. vivax* can be found in a wider geographical distribution than *P. falciparum*, simply because of its ability to develop at lower temperatures in the vector hosts (Igweh, 2012; WHO, 2016a). *Plasmodium vivax* can also survive in cooler climate and high altitudes. The parasite's dormant liver stage (hypnozoite) enables it to survive for longer periods and becomes reactivated as soon favorable factors return and it reinvades the blood (Igweh, 2012). *Plasmodium ovale* is mostly found in West Africa and Western Pacific Islands. *Plasmodium ovale* is morphologically similar to *P. vivax*, but it can infect Duffy negative individuals unlike *P. vivax. Plasmodium malariae* has a quartan cycle (three-day cycle), unlike other human malaria parasites and can cause chronic infections and often asymptomatic with low parasitaemia (Garnham, 1966). *Plasmodium knowlesi* is difficult to diagnose by microscopy and hence, often miss-diagnosed as *P. malariae* (Kantele & Jokiranta, 2011; Singh et al., 2004). *Plasmodium knowlesi* is known to be a natural malaria parasite of long-tailed macaque monkeys, but recent evidences have shown that it is a significant cause of zoonotic malaria across Southern east Asia (Cox-Singh et al., 2008; Figtree, 2010; Kantele & Jokiranta, 2011; Singh et al., 2004; Vythilingam & Hii, 2013; Vythilingam et al., 2008; Vythilingam et al., 2006).

Furthermore, non-human primates have been used extensively in the laboratory for biological, immunological and chemotherapeutic studies on human malaria parasite, *P. falciparum*, while they have their own pathogenic malaria parasites such as *P. knowlesi*, *P. coatneyi*, *P. fragile*, *P. cynomolgi*, *P. inui*, *P. fieldi*, *P. simiovale* and *P. gonderi*. These are mostly parasites of old world monkeys, except *P. brasilianum* and *P. simium* which are parasites of new world monkeys (Collins, 2002).

There are 4 common malaria parasites that are infectious to rodents. These parasites include *P. berghei*, *P. vinckei*, *P. chabaudi* and *P. yoelii*. The virulence of these rodent malaria parasites depends on factors such as; species and strains of parasite, species and strains of rodents, sex and age of the host (Amani et al., 1998; Sanni et al., 2002), hence their suitability for experimental malariology. It is noteworthy that most laboratory strains of rodent malaria parasites have been isolated from African Thicket rats, *Thamnomys rutilans* (Landau & Boulard, 1978), and thus, the process of natural selections does not occur between the parasite and the laboratory host unlike human malaria parasites. *Plasmodium berghei* prefers to infect both matured red blood cells (RBCs) and young red blood cells (reticulocytes) and can serve as experimental model for pathogenesis of cerebral malaria (CM) (Neill & Hunt, 1992; Sanni et al., 1998). *Plasmodium yoelii* mostly prefers to parasitize reticulocytes, except for *P. yoelii* YM (lethal) which can infect both matured RBCs and reticulocytes and mostly used as experimental model for malaria vaccines (De Souza et al., 1997; Sanni et al., 2002). On the other hand, *P. vinckei* mainly parasitizes matured RBCs and is useful in studying chemotherapy, immune mechanisms and pathogenesis of malaria (Kremsner et al., 1992), while *P. chabaudi* mostly prefers matured RBCs and suitable to study immune mechanisms and pathogenesis of malaria (Cross & Langhorne, 1998).

2.1.3 Life cycle of malaria parasite

Plasmodium is transmitted by female *Anopheles* mosquitoes. The life cycle of malaria parasite is complex but it is similar from one species to another, other than differences in days of development. Sporozoites from the saliva of an infected female mosquito are injected into the blood capillaries of a vertebrate host during blood meal (Figure 2.1). These sporozoites then circulate in the blood system and are taken to the liver (Ponnudurai et al., 1991), where it undergoes pre-erythrocytic schizogony. Once in the liver, it invades and replicates in the hepatocytes (Pradel et al., 2002) and transforms into thousands of

merozoites in the liver. At the end of pre-erythrocytic schizogony, the schizont bursts and releases merozoites into the blood circulation. This pre-erythrocytic or exo-erythrocytic stage, takes up to 48-68 hours in the rodent parasites (Bafort, 1968; Landau & Killick-Kendrick, 1966; Yoeli et al., 1965a; Yoeli et al., 1965b) or can be as long as 15 days in *P. malariae* in humans (Igweh, 2012). *Plasmodium vivax* and *P. ovale* have a latent or dormant form (hypnozoite), which can remain dormant in the liver for weeks or years, and often, the dormant schizonts are reactivated and release merozoites, causing clinical relapse (Igweh, 2012).

The erythrocytic stage begins with the merozoites invading the erythrocytes. They grow and multiply through ring, trophozoite and schizont stages (erythrocytic schizogony). The erythrocytic schizont bursts and merozoites are released, after which they invade new erythrocytes. However, some newly invaded merozoites differentiate into sexual forms, microgametocyte (male form) and macrogametocyte (female form). It takes some days after post infection for the gametocytes to appear in the blood, which varies according to the species of the parasite. It takes about 7-15 days post infection for the *P. falciparum* gametocytes to appear in the blood stream, while it takes as early as 1-3 days in other species (Igweh, 2012). The gametocytes have 2-3 days half-life, while they may persist up to weeks in some (Bafort, 1968; Yoeli et al., 1965a). The gametocytes are infectious to *Anopheles* female mosquitoes and the mosquitoes later pick up the gametocytes during blood meal.

In the mosquito midgut, the gametocytes develop into gametes and fertilize each other, to form motile zygotes, known as ookinetes. The ookinetes penetrate and escape into the gut membrane and come to lie beneath the basement membrane and form oocysts within which they multiply to produce numerous sporozoites. The mature oocyst ruptures releasing sporozoites into body cavity and migrate to the salivary glands where they are

ready to be injected into the next available vertebrate host through bites during blood meals. The life cycle begins again after successful bites on a susceptible vertebrate host by infected mosquitoes.

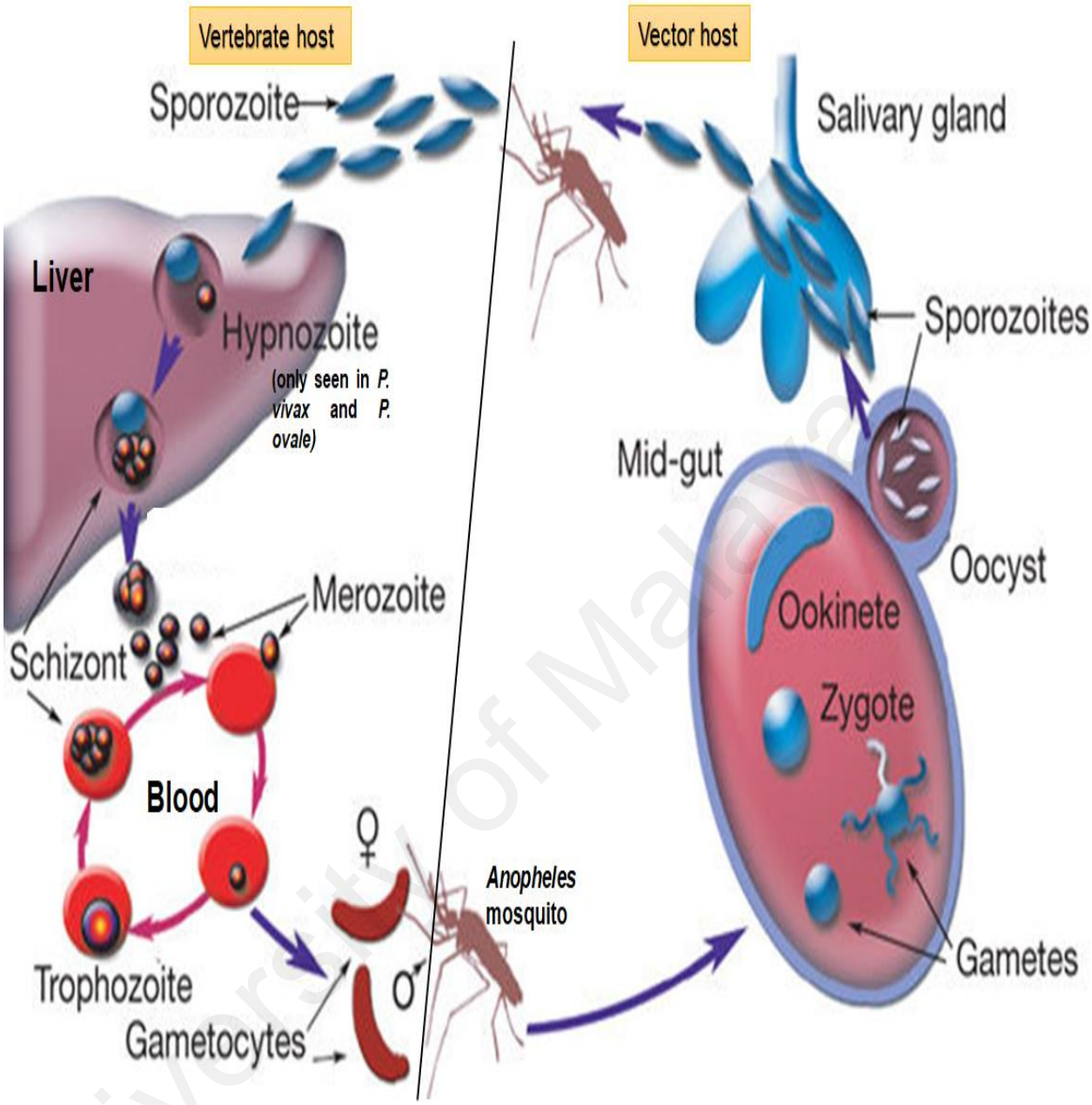


Figure 2.1: Life cycle of malaria parasites (adapted from Winzeler (2008)).

2.2 IMMUNOLOGY OF MALARIA

The pathogenesis of malaria is initiated at the erythrocytic stage of the life cycle, with clinical symptoms such as fever, hypoglycemia or complications such as acidosis and anaemia, as a result of severe malaria. Immunity to malaria is complex, as the parasite has multiple life-stages and a large genome, with around 5000 genes (Beeson et al., 2008) of potential immune targets. Protective immunity requires repeated exposure to malaria in order to generate an effective adaptive response. Adaptive immunity might be difficult to attain, due to the fact that the parasite has some proteins encoded by *var* genes which undergo frequent non-homologous recombination and this leads to heterologous antigenic variation (Artavanis-Tsakonas et al., 2003).

The migration of sporozoites from the blood to the liver is mostly asymptomatic (Zheng et al., 2014), but reports have shown that immunological responses are elicited and the parasite has also evolved mechanisms to evade these responses (Gomes et al., 2016). However, it has been estimated that about 20-30% of sporozoites that enter the lymphatic system are hindered at the proximal lymph node, where dendritic cells (DCs) help in killing of the parasites (Amino et al., 2006; Zheng et al., 2014). Also, inflammatory cytokines such as type 1 interferons (IFN) have been identified to inhibit the growth of the liver form (Liehl et al., 2014). Similarly, reports have shown that innate immune cells such as Natural Killer (NK), Natural killer T cells (NKT) and γδT cells mostly inhibit the development of exoerythrocytic forms (McKenna et al., 2000; Roland et al., 2006). Inside the liver, the Kupffer cells (KC) are known to be immunophagocytic cells which are often responsible for killing microorganisms invading hepatocytes. However, sporozoites migrate through these KC without being halted (Zheng et al., 2014). Sporozoites do not only migrate through these KCs, they also induce the apoptosis of KC (Klotz & Frevert, 2008; Tavares et al., 2013) in the process, and thus, it has been

established that sporozoites contact with KCs downregulates the inflammatory cytokines such as tumour necrotic factor-α (TNF-α), IL-6 and monocyte chemoattractant protein 1 (MCP-1), and thereby upregulates the anti-inflammatory cytokine, IL-10 (Klotz & Frevert, 2008). So, the immune modulatory activities of the sporozoites secure their passage and survival to merozoites and continue to grow at the erythrocytic stages.

Furthermore, the invasion and multiplication of *Plasmodium* in red blood cells (RBCs) alter the shape, size, density and permeability of the erythrocytes (Kyes et al., 2001). Matured RBCs lack the ability to express major histocompatibility complex (MHC) class I and II, or other necessary antigen-processing machinery, hence, $CD8^+$ T cells and $CD4^+$ T cells cannot directly target infected RBCs (iRBCs) or extracellular merozoites. So, the reduction or elimination of the parasites is at the liberty of antibody-dependent mechanisms and other phagocytic cells. Antibodies (Abs) can bind to iRBCs, thereby initiating their phagocytosis through opsonization by macrophages (Gomes et al., 2016; Osier et al., 2014). Abs also bind to extracellular merozoites, forming membrane attack complex and inhibit the invasion of RBCs (Boyle et al., 2015).

Several reports have shown that antibodies target merozoite surface protein (MSP)-1 for growth inhibition (Daly & Long, 1995; Egan et al., 1999; John et al., 2004; Stowers et al., 2002). Also, merozoite proteins such as apical membrane antigen (AMA) -1 and erythrocyte-binding antigen -175 (EBA -175) are involved in RBC invasion and have been identified to be targeted by antibodies (Ford et al., 2007; Mlambo et al., 2006).

The type of antibodies produced during malaria infection is influenced by the innate immune response surrounding the B-cell subsets (Deroost et al., 2016; Leoratti et al., 2008). Cytophilic Ab such as immunoglobulin G (IgG) 1 and IgG3 have been associated with lower parasitaemia or lower risk of malaria attack in endemic areas (Sarthou et al., 1997; Tangteerawatana et al., 2001), while IgG4 which compete with the effector

mechanisms of the cytophilic Ab is considered non-protective (Garraud et al., 2003). The predominance of cytophilic IgG1 and IgG3 during malaria infection have been attributed to the elevation of proinflammatory Th-1 cytokines (Deroost et al., 2016). Nevertheless, the Ab protection against malaria infection in human has been reported to be short-lived and requires persistence or repeated exposure, to confirm long term immunity on the host (Ryg-Cornejo et al., 2016).

In addition, the role of T-cells in the clearance or reduction of parasitaemia is indispensable. $CD4^+$ T-cells have been identified to play key roles in protection against erythrocytic stages, while contributions from $CD8^+$ T-cells and γδ T-cells are minimal (Taylor-Robinson, 2010). Evidence from *in vitro* experiment shows that $CD4^+$ T-cells from mice and humans, proliferate and produce IFN-γ and interleukin (IL)-4, when stimulated (Van der Heyde, 1997). The early stage of malaria infection is predominated by elicitation of IFN-γ (Th-1 cell), while there is a shift to Th-2 cell, IL-4 at later stage of the infection (Taylor-Robinson & Smith, 1999). In a murine malaria model, the injection of recombinant IFN-γ was discovered to protect against a lethal *P. yoelii*, while neutralization of IFN-γ in a non-lethal *P. chabaudi* model, exacerbates the disease outcome (Favre et al., 1997; Li et al., 2001; Meding et al., 1990; Shear et al., 1989). Hence, the role of IFN-γ in controlling parasitaemia has long been established. IFN-γ also helps in induction of proinflammatory cytokines such as tumor necrotic factor (TNF)-α, IL-1 and IL-6, presumptively through macrophages activation (Li et al., 2001; Taylor-Robinson, 2010). Arguably, IFN-γ can be said to be the determinant of all immunological pathways associated with protection from malaria disease (McCall & Sauerwein, 2010).

Nonetheless, innate immune system plays significant role as first line of defence mechanism. Parasite by product such as hemozoin from ruptured iRBCs, has been shown to stimulate dendritic cell maturation, and thereby induces innate immunity (Coban et al.,

2002). Thus, the ligation of toll-like receptor 9 activates innate effector mechanisms (Coban et al., 2005). Studies on mice have suggested that early production of IFN-γ in naïve mice can be the result of activation of innate immune system such as macrophages, NK cells or phagocytic granulocytes (Artavanis-Tsakonas & Riley, 2002; De Souza et al., 1997; Mohan et al., 1997). More so, it has been suggested that the strong IFN-γ early response stimulated by NK cells might be to restrict replication of the parasite and hence, avoid hyper parasitaemia and severe pathology (Artavanis-Tsakonas et al., 2003).

2.3 PATHOGENESIS OF SEVERE MALARIA

Severe malaria can be described as a complex multisystem disorder with various syndromes, which includes severe malaria anaemia (SMA), cerebral malaria (CM), placental associated malaria (PAM), renal failure, metabolic acidosis, hypoglycemia and acute respiratory distress (ARD) syndrome (Mackintosh et al., 2004; Schofield, 2007). In the process of the host controlling or eliminating malaria parasite during infection, imbalances in the immune responses often lead to severe pathology (Deroost et al., 2016; Lamb et al., 2006). It is important to note that factors such as age, immune status, transmission rate, gender and host genetic background, play important roles in determining disease severity. However, the most fatal cases of severe malaria are mostly caused by SMA, ARD syndrome, and CM, which often overlap during the course of infection (Deroost et al., 2016; Langhorne et al., 2008; Marsh et al., 1995).

Most studies on human and animal malaria infections have implicated the nature of immune responses, mostly the proinflammatory pathways as the leading cause of pathology (Lamb et al., 2006). It has also been suggested that the production of a potent and rapid innate immune response can elevate the pathology of severe malaria (Riley, 1999). Animal studies have shown that over production of IFN-γ, TNF/LT-α and IL-12, had been identified to have pathogenic role in *P. berghei* infection (Engwerda et al., 2002;

Yoshimoto et al., 1998). In *P. c. chabaudi* infection, elevation of IFN-γ and TNF had been associated with pathology, while regulatory cytokines such as IL-10 and TGF-β ameliorate the disease condition (Li et al., 1999; Li et al., 2003; Linke et al., 1996). Similarly, high level of serum TNF-α and low level of TGF-β1 and IL-12 have been associated with severe malaria, mostly SMA and CM in children (Kwiatkowski et al., 1990; O'Garra & Arai, 2000). High risk of fever and severe malaria have been correlated with high ratios of TNF-α, IFN-γ, and IL-12 to TGF-β (Cai et al., 1999). Likewise, profiling the serum level of cytokines of Malian children with severe *falciparum* malaria showed that, IL-6 and IL-10 were elevated significantly for CM cases, while SMA cases were associated with lowered IL-6 and IL-10, and hyper-parasitaemia cases experienced declined IL-6 response (Lyke et al., 2004).

Furthermore, parasite burden is among the factors to be considered in pathogenesis of malaria. The accumulation of parasites in various organs often leads to mechanical blockage of blood vessels and invariably, leading to organ-specific syndromes such as CM, malaria associated ARD syndrome, PAM and acidosis (Brugat et al., 2014; Hunt et al., 2014; Hunt et al., 2006; Schofield, 2007; Schofield & Grau, 2005). Cerebral malaria has been characterized with high parasite load in other organs apart from the brain, which include spleen, lungs, kidneys, skin and intestine (Milner et al., 2015; Milner et al., 2014; Seydel et al., 2006). Conversely, clinical studies on CM have shown that the brain microvasculature may have sequestered iRBCs only or sequestered parasites with haemorrhages, accumulation of pigmented leukocytes and thrombi or no sequestration of iRBCs (that is coma or death resulted from other causes) (Milner et al., 2013; Milner et al., 2014; Taylor et al., 2004). Thus, this implies that there are complex and multiple mechanisms of pathogenesis that lead to CM.

Studies have shown that young children and pregnant women are the most vulnerable to life-threatening anaemia (Siqueira et al., 2015). Several reports from human and animal studies have suggested that the mechanisms underlying the pathogenesis of SMA may include the clearance and/or destruction of iRBCs (Wildig et al., 2006; Yap & Stevenson, 1992); clearance of uninfected RBCs (Chang et al., 2004; Evans et al., 2006; Jakeman et al., 1999); reduction in RBCs production (erythropoietic suppression) and dyserythropoiesis (Ghosh & Ghosh, 2007; Lamikanra et al., 2007; Pathak & Ghosh, 2016). More so, some studies have demonstrated that high ratio of TNF-α /IL-10 is associated with SMA in both human and animal models (Kurtzhals et al., 1998; Othoro et al., 1999) and polymorphisms in human TNF-α showed more association with anaemia than CM (McGuire et al., 1999). Similarly, TNF-α neutralization *in vivo* reversed anaemia displayed by IL-10 knock-out mice infected with *P. chabaudi* (Li et al., 2003; Linke et al., 1996). Furthermore, PAM has been attributed to the sequestration of parasites in the placenta, which often result in maternal anaemia, low birth weight, abortion, still birth or death (Ismail et al., 2000; Nosten et al., 1999; Rogerson et al., 2003).

In conclusion, *Plasmodium* infection is associated with strong inflammatory responses and sequestration (common with *P. falciparum* in human and *P. berghei* in rodents) in many organs, thereby inducing pathogenesis and /or histopathology in organ-specific manner. Therefore, it can be suggested that multiple pathways or mechanisms might be responsible for production of immune response that leads to severe malaria. Hence, there is need for more evidences or researches to determine the definitive host response which are protective or pathogenic.

2.4 FILARIASIS

2.4.1 Filaria infection

Filariasis is a parasitic disease caused by filaria nematodes. There are several filaria species that infect humans and animals. These include; *Wuchereria bancrofti*, *Brugia malayi*, *B. timori*, *Onchocerca volvulus*, *Loa loa*, and *Mansonella* spp, which mostly infect humans, while *B. pahangi*, *Dirofilaria immitis* and *D. repens* are major threat to the canines. Among these parasites, *W. bancrofti*, *B. malayi* and *B.timori* are the causes of lymphatic filariasis (LF) which affect over 67 million people in about 73 countries (Ramaiah & Ottesen, 2014; WHO, 2016a). Lymphatic filariasis is the leading cause of permanent disability in the world, which accounts for about 19.43 million hydrocoele cases, lymphoedema (16.68 million cases) and disability-adjusted life years (2.02 million) (Dickson et al., 2017; Murray et al., 2015). *Wuchereria bancrofti* which is predominant in Africa and patchy in other parts of the world, is responsible for about 90% cases of LF, while *B. malayi* is dominant in Asia and patchy in other parts of the world, is responsible for the remaining 10% (Bockarie et al., 2009).

Generally, bancroftian filariasis is transmitted by *Culex quinquefasciatus* (85%), *Anopheles* spp (9%), *Mansonia* spp (5%) and the remaining 1% by *Aedes*, *Ochlerotatus* and *Downsiomyia* (Bockarie et al., 2009; Manguin et al., 2010; Zagaria & Savioli, 2002). On the other hand, brugian filariasis is mainly transmitted by *Mansonia* and *Anopheles* spp (for *B. malayi*), and *An. barbirostris* is the vector for *B. timori* (Manguin et al., 2010).

2.4.2 Filaria nematodes

Filariae are round worms or nematodes, which dwell in the host tissues. The filaria worms belong to the class Secernentea, order Spirurida, superfamily Filaroidea and family Onchocercidea. The filaria worms undergo a developmental stage in an intermediate host, mainly arthropods. The adult filaria nematodes dwell in various tissues

such as lymphatics, skin nodules, subcutaneous and pleural cavity (depending on the species), while the microfilariae (mf) are released into the blood stream or under the skin, as in the case of *O. volvulus*. The species of mf are being differentiated by the sheath, cephalic space and the number and position of caudal nuclei (Anderson, 2000).

Wuchereria bancrofti and *B. malayi* which cause LF, are the most widely distributed and prevalent, amongst all the filaria nematodes that infect humans. *Wuchereria bancrofti* and *B. malayi* often coexist in many places and can co-infect man (Dickson et al., 2017; Manguin et al., 2010). Due to the periodicity of the circulating mf, there are 3 variants of *W. bancrofti*; nocturnal periodic (NP) form, nocturnal sub-periodic (NSP) form, and diurnal sub-periodic (DSP) form, while *B. malayi* has NP and NSP forms, and *B. timori* only has NP form (Manguin et al., 2010; Paily et al., 2009).

Microfilaria periodicity is the time or period at which the mf will be present in peripheral blood or circulation. In NP forms, the peak of mf periodicity is 2200 – 0300 h while in NSP forms, the mf will be present 24 h in the peripheral blood but will peak at 1800 – 2000 h (Gould et al., 1982; Paily et al., 2009). Different hypotheses have been highlighted as the mechanism behind mf periodicity. It has been suggested that absent or low mf count in the peripheral blood during the day, can be attributed to more oxygen tension in the lungs during the day when compared to that in the night, and hence, accumulation of mf in the lungs during day (Grove, 1990; Hawking et al., 1981). Stimuli such as host body temperature has been identified to modulate the mf periodicity (Hawking et al., 1966). In addition, it has been suggested that the circadian rhythm of mf is oriented towards the circadian rhythm of the host, and hence, mf periodicity coincides with the vector's feeding behavior (Buck, 1991; Vanamail & Ramaiah, 1991; Weerasooriya et al., 1998). This action exposes the vector mosquitoes to a high and ready circulating mf and thus, aids in successful transmission. However, NP *W. bancrofti* is the

most widely distributed variant across tropical Africa, Middle East, Southern Asia, Papua New Guinea and Western Pacific Island (Michael & Bundy, 1997; WHO, 2008), due to availability of various mosquito species as vectors which are present in both rural and urban areas (Mak, 1987). The NSP form of *W. bancrofti* is restricted to mainly Thailand along its border with Philippine, Myanmar, Malaysian Borneo and Northern Vietnam (Meyrowitsch et al., 1998; Pothikasikorn et al., 2008), while DSP form is restricted to the islands of South-Western Pacific and India Ocean (Manguin et al., 2010; Paily et al., 2009).

Another filaria worm which is of threat to human is *Onchocerca volvulus* causing onchocerciasis, also known as river blindness. It is transmitted by the vector, *Simulium* also known as black fly. The disease is widely prevalent in about 30 countries in Africa (accounted for 99% cases) and distribution in central and South America, and part of Yemen is patchy (Boatin et al., 1997; WHO, 1995; Zouré et al., 2014). The adult *O. volvulus* dwells in the skin nodules, while the mf are found mainly in the skin (Paily et al., 2009). The *Onchocerca* mf in the skin often migrates through the intercellular fluid to the eye, inducing an inflammatory reaction which can lead to blindness (Boatin et al., 1997; Roberts LS, 1996). Depigmentation and thickening of the skin often result from the presence of mf in the skin (WHO, 1995). Genital elephantiasis and hanging groin are amongst other clinical manifestations associated with onchocerciasis. In addition, loasis is caused by *Loa loa*, also known as African eye worm, and it is transmitted by *Chrysops*. It is mainly found in the equatorial rain forest regions of West and Central Africa (Remme et al., 2008; Zouré et al., 2014). Its clinical manifestations include severe itching, oedema or calabar swellings and joint pains (Boussinesq, 2006; Melrose, 2004).

Furthermore, human infection with *Mansonella* is of low prevalence in most endemic regions and it is mostly restricted to many parts of sub-Sahara Africa and patchy in South

America (Simonsen et al., 2014; Simonsen et al., 2011). The adults of *M. ozzardi* and *M. perstans* live in the serous body cavity, while their mf are found in the blood streams (Downes & Jacobsen, 2010; Paily et al., 2009). On the other hand, *M. streptocerca* is regarded to be aparasitaemic mainly because both the adults and mf are found in the skin (Crainey et al., 2016; Simonsen et al., 2011). The vectors of these parasites are biting midges (*Culicoides*) and also, blackflies (*Simulium*) have been identified to transmit *M. ozzardi* in Northern Argentina (Shelley & Coscarón, 2001).

Brugia pahangi is a closely related species of *B. malayi* (Lau et al., 2015), which naturally infect cats, dogs and wild carnivores (Denham & McGreevy, 1977). However, there are reports which showed evidence of natural infections of *B. pahangi* in humans, for example in Borneo, Indonesia (Palmieri et al., 1985). Experimental infection in human with *B. pahangi* shows similar symptoms to that of LF (Edeson et al., 1960). Also, Tan et al. (2011) gave an account of clinical cases of zoonotic *B. pahangi* filariasis in a suburbia of Kuala Lumpur city, Malaysia. Unlike other *Brugia* species, *B. pahangi* has been used extensively in animal experimental model for filariasis (Ash, 1973; Ash & Riley, 1970; Lau et al., 2015; Porthouse et al., 2006; Vincent et al., 1980).

Furthermore, other zoonotic filaria worms include *D. immitis* and *D. repens*. *Dirofilaria immitis* is a filaria nematode that causes heartworm disease in dogs but has been reported to occasionally infect humans (Rodrigues-Silva et al., 1995), while *D. repens* was previously reported to cause lesions in the lungs, subcutaneous nodules and breast lumps in humans (Bennett et al., 1989; Pampiglione et al., 1995). Although dirofilariasis in humans are rare cases, it is mostly asymptomatic and the worms are trapped and calcified in the lungs, resulting in a carcinoma-like lesions (Melrose, 2004). In addition, filaria species of animals such as *Brugia beaveri* (raccoons), *Mansonella interstitium* (squirrels), *Meningonema peruzzi* (monkey), *Mansonella radhaini*

(chimpanzee), *Microfilaria bolivarensis* and *Microfilaria semi-clarum*, have all been implicated in zoonotic filariasis (Grove, 1990; McMahon & Simonsen, 1996; Melrose, 2004; Orihel & Eberhard, 1998; Petrocheilou et al., 1998).

2.4.3 Life cycle of filaria nematodes

Filariae make use of 2 different hosts to complete their life cycle (Figure 2.2). The definitive host where the parasite undergoes sexual reproduction, takes place in human or other vertebrate hosts, while in the intermediate host, the parasite undergoes maturation and mostly occurs in biting or blood sucking arthropods such as black flies, mosquitoes and midges.

In LF, the female mosquito ingests mf while feeding on an infected definitive host. The microfilariae penetrate the abdominal wall and migrate to the thoracic flight muscles, where they differentiate and molt from larvae 1 (L1) to L3. Usually, this migration occurs within 24 h and exsheathment or shedding of the mf sheath takes place in the midgut and hemocoele (Chen & Shih, 1988; Paily et al., 2009). The L1 molts into L2 within 5-7 days, and by day 10-14 (depending on temperature and other factors), it molts into L3, which is a very active and infective stage. At this stage, the L3 breaks through the flight muscles into the hemocoele and migrates to the head region, near the labium of the proboscis, where it is ready to be transmitted into the next available human host. The L3 will remain alive in the mosquito, for as long as the mosquito survives (Paily et al., 2009; Paily et al., 1995).

When the infected mosquito feeds on a human host, L3 is deposited on the host skin via the labium, and the L3 enters the host through mosquito bite wound or nearest opening or breaks in the skin. Successful transmission is aided by high ambient humidity and skin moisture (Manguin et al., 2010). Upon entering the host body, the L3 travels through the lymphatic vessels to lymph nodes, where development begins. This is then followed by

two intermediate molts (L3 - L4 - L5) into adult male or female worms. The period of appearance of mf in the peripheral blood (known as patency period) varies from species to species and hosts. It takes about 4 to 15 months or more for *W. bancrofti* mf to appear in peripheral blood (Manguin et al., 2010), while it takes about 41 - 46 weeks to detect *B. malayi* mf in experimental infection of man and the adult female can produce mf for over 8 -9 years (Wang et al., 1994). Whereas, it takes about 76 to 209 days for *B. malayi* and 56 to 138 days for *B. pahangi* to produce mf in gerbils (Ash, 1973).

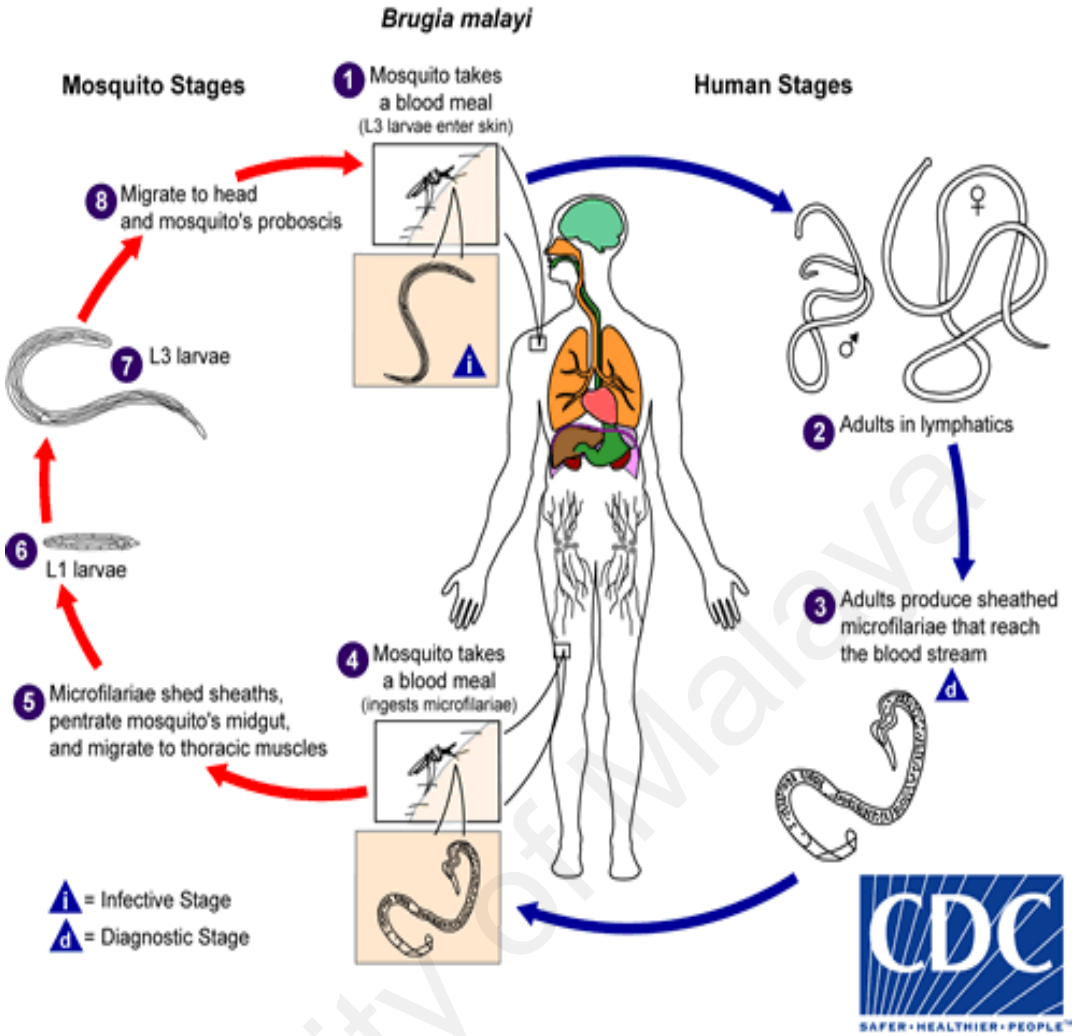


Figure 2.2: Life cycle of a lymphatic filaria parasite.

During a blood meal, an infected mosquito introduces third-stage filaria larvae (L3) onto the skin of the host and enter into the bite wound (1). They develop into adults that mostly reside in the lymphatics (2). Adults produce microfilariae which are sheathed and have nocturnal periodicity. The microfilariae migrate into lymph and enter the blood stream reaching the peripheral blood (3). Mosquitoes ingest the microfilariae during blood meal (4). After ingestion, the microfilariae lose their sheaths and migrate through the wall of the midgut to reach the thoracic muscles (5). Here, the microfilariae develop through first stage larvae (L1) (6) to third-stage larvae (L3) (7). The third-stage larvae migrate through the hemocoel to the mosquito's proboscis (8) and can infect another human when the mosquito takes a blood meal (1). Source: (CDC).

2.5 IMMUNOLOGY OF FILARIASIS

Filariasis in an endemic community has been categorized into three: exposed individuals who show no signs of pathology or infection (resistant or endemic normal); individuals with substantiated clinical symptoms such as elephantiasis, lymphoedema and river blindness; and individuals with mild infection with circulating mf or parasite antigen (Maizels et al., 1995; Metenou & Nutman, 2013; Ottesen, 1995; Pfarr et al., 2009). Hence, it is on these basis, clinical manifestations are being reflected. The asymptomatic (endemic normal) individuals elicit a well-balanced Th1/Th2 immune response through suppression of IFN-γ and IL-5 (Sartono et al., 1997) and elevated IL-10 (Mahanty et al., 1996), while the tolerant individuals (mild infection with circulating mf) show modified Th2 immune response with low level of Th1 and high level of IL-10, and individuals with obvious clinical symptoms elicit uncontrolled Th1 and Th17 responses (Babu et al., 2009), with high levels of IgE isotype but low IgG4 response (Maizels & Yazdanbakhsh, 2003).

In filaria animal studies, L3 elicits a significant level of IL-4 responses irrespective of the infection route, at early stage of infection (Lawrence et al., 1994; Lawrence & Devaney, 2001; Osborne & Devaney, 1998). Infective larva primes the immune responses for optimum production of IL-4 while growing to adult stage in the host, and this was observed in the implantation of adult worms in BALB/c mice (Lawrence et al., 1994). The role of T cells has been demonstrated to differentiate into IL-4 producing cells when exposed to macrophages from the peritoneal cavity of adult *B. malayi* implanted mice (Lawrence & Devaney, 2001; P'ng Loke & Allen, 2000). The significant role of IL-4 has been proven beyond doubt in human studies which shows no difference in the levels of IL-4 elicited by all categories of patients in endemic area (Dimock et al., 1996; Maizels et al., 1995).

Furthermore on animal studies, infection of BALB/c mice with *B. pahangi* L3 results in elevated levels of IL-4, IL-5 and IL-10, while neutralizing splenic cells from the same mice with anti-IL-4 or recombinant IL-12 shows proliferation of IL-2 and IFN-γ production (Osborne et al., 1996). Study by Osborne and Devaney (1998) demonstrated L3 of *B. pahangi* to elicit IL-4 in the lymph node of infected gerbils, while mf stimulate increased levels of IFN-γ mRNA in the absence of IL-4 or IL-10 induction, and that the source of early production of IL-4 is from double negative T cell ($CD4^-$ $CD8^-$ αβ T cells). The same authors (Osborne & Devaney, 1999) further established IL-10 and antigen presenting cells (APCs) play major role in suppressing Th1.

Moreover, modulation of the immune responses in filariasis is somewhat complicated and complex due to exposure of the host to different life cycle, difference in intensity of infection, host genotype and presence of secondary infections (Ottesen, 1992). It has been suggested that during chronic helminth infections, Th2 effector cytokines such as IL-5 and IL-13 are down regulated, while Th2 regulatory cytokines such as IL-4 and IL-10 continue to proliferate (Maizels et al., 2004), and this has been attributed to its potential to cause fibrosis (Chiaramonte et al., 1999). Thus, it can be suggested that immunomodulation by filaria worms is somewhat beneficial to both the host and parasite. This is because hyper-responsiveness will kill or reduce the parasite loads, but may lead to pathology (Dreyer et al., 2000). Severe dermatitis in case of onchocerciasis has been attributed to excessive proliferation of mast cells and eosinophils which kill the mf, but in the process, causes the pathology (Korten et al., 1998). In addition, patients with skin lesions due to onchocerciasis has been related to highly skewed Th2 responses and absence of immunoregulators (Timmann et al., 2003). Conversely, hypo-responsiveness to filaria worms allows mf loads without consequential pathology (McSorley & Maizels, 2012; Wildenburg et al., 1996).

Overall, the acute phase of filariasis has been associated with cytokines such as IL-5, IL-4, IL-2, IFN-γ and IL-13, and antibody isotype IgE, while the chronic phase of the infection is known with elevated regulatory cytokines such as IL-10 and TGF-β, and antibody isotype IgG4 (King & Nutman, 1993; Kwan-Lim et al., 1990; Metenou & Nutman, 2013; Soboslay et al., 1997). Several studies have demonstrated the role of IL-10 and TGF-β in immunomodulation of the host response by neutralizing antibodies to IL-10 and TGF-β which in turn, show reversed unresponsiveness of the T cells and cytokine production (Babu et al., 2006; King et al., 1993; Steel & Nutman, 2003) and also, antigen stimulation of filaria infected individuals produce high level of IL-10 in *ex vivo* (Mahanty et al., 1996; Mahanty & Nutman, 1995). Similarly, mice treated with neutralizing antibody to IL-10 or mice deficient in IL-10 showed lower mf in *B. malayi* infection when compared with wild type mice (Simons et al., 2010).

Furthermore, there are evidences to show that modulatory cytokines, IL-10 and TGF-β may also regulate APCs function (Korten et al., 2011; P'ng Loke et al., 2000; Taylor et al., 2006). This has been buttressed with the studies which showed patent filaria infection is being associated with IgE (Kwan-Lim et al., 1990; Metenou & Nutman, 2013; Simonsen et al., 1996; Soboslay et al., 1997). Concurrently, animal studies have also shown the effect of modulating cytokines on APCs with filaria infected animals showing T cell unresponsiveness (Gillan et al., 2005; MacDonald et al., 1999; Osborne & Devaney, 1999; Taylor et al., 2006).

2.6 PATHOGENESIS OF FILARIASIS

The pathology of parasitic infection is a function of the parasite, host and environment, and their interactions. Often times, the host mounts cellular and molecular response against the parasite, while the parasite has also evolved mechanism to continue its survival and production (Mak, 2012). Hence, for the host-parasite interactions not to lead

to pathology, it is imperative for the host to launch and maintain an effective response (Anthony et al., 2007).

The developing and adult worms of filaria nematodes responsible for LF dwell in the lymph nodes and afferent lymphatics, whereas their larvae (mf) circulates in the blood. Damage to the lymphatics begins with vessel dilation, aided by the adult parasite secretions and in due course leads to impairment of lymphatic contractility (Chakraborty et al., 2013). As the disease progresses, the dilated vessels experience stasis of lymph due to compromised unidirectional valves (Olszewski, 2002); while further lymphatic damage is caused by secondary bacteria or fungal infections which often leads to lymphoedema, hydrocele and elephantiasis (Bennuru & Nutman, 2009; Chakraborty et al., 2013; Nutman, 2013; Shenoy, 2008). However, it has been suggested that living adult worm, dead worm and its inflammatory responses, mf and secondary pathogenic infections, are the major factors that determine the clinical manifestation of LF (Bennuru & Nutman, 2009; Chakraborty et al., 2013).

The living adult worms have been associated with subclinical lymphangiectasia and it has also been observed in children *in vivo* (Dreyer et al., 1999b; Figueredo-Silva et al., 2002). It has been suggested that living adult worms invoke a transient inflammatory response and distended lymphatic vessels (Dreyer et al., 1999b). However, the extension of the lymphangiectasia beyond the worm's niche, suggest that the parasite secretions or excretions act on the lymphatic endothelial cells (Figueredo-Silva et al., 2002; Nutman, 2013).

Moreover, the adult filaria worms may die due to many reasons (drugs or host related), but irrespective of the cause of death, the dead worms elicit inflammatory reactions (Figueredo-Silva et al., 1996; Figueredo-Silva et al., 2002). Several reviews have suggested that immune response targeted at the dead worms, often leads to lymphatic

blockage and hence, gross pathological lesions and decalcification of adult worms (Bennuru & Nutman, 2009; Figueredo-Silva et al., 2002; Nutman, 2013). This has been attributed to damage to the lymphatic valves as a result of prolonged lymphatic dilatations, which in turn causes lymphatic back flow and lymphoedema (Bennuru & Nutman, 2009). However, this lymphatic dysfunction often predisposes patients to secondary pathogenic infections and stimulate inflammatory responses, mostly in the subcutaneous and skin which then progresses to lymphoedema and elephantiasis (Nutman, 2013; Olszewski et al., 1997; Shenoy, 2008). The alterations in the structure of the lymphatics also referred as lymphatic remodeling, is a result of endothelial cell growth, cell proliferation, migration and distorted extracellular matrix and it's consequential from lymphangiectasia to granuloma formation (Bennuru & Nutman, 2009; Chakraborty et al., 2013; Witte et al., 1997).

Nevertheless, the disease manifestation of LF goes beyond the lymphatic systems. Extra-lymphatic diseases associated with bancroftian or brugian filariasis are mainly caused by mf, unlike the lymphatic disease (Dreyer et al., 1999a). Among the extra-lymphatic filaria disease identified are; filaria arthritis and filaria pseudo-rheumatism (Ismail & Nagaratnam, 1973; Panda et al., 2013; Pineda et al., 2012; Weinberger et al., 1979), intra-articular deposition of immune complexes (Dreyer et al., 1999a); tropical pulmonary eosinophilia (Boggild et al., 2004; Dreyer et al., 1996; Magnussen et al., 1995) caused by pulmonary infiltrates and eosinophilia; and renal disease associated with glomerulonephritis, hematuria and proteinuria (Dreyer et al., 1999a; Nayak et al., 2011; Suzuki et al., 2001). More so, the gamma-glutaryl transpeptidase found on human pulmonary epithelium is similar to that present in the L3 of *B. malayi* (Lobos et al., 2003; Vijayan, 2007).

Generally, the response of the host to an invading parasite is very important in determining the manifestation of the disease that follows. It has been suggested that natural killer (NK) cells aid in down regulating the host immune response during *B. malayi* infection, and hence provides trophic factors which support the parasite growth (Babu et al., 1998). In addition, report on *B. malayi* infection in cats has shown that both live and dead worms can induce inflammatory responses, dilatations and valvular thickening (Rogers & Denham, 1974). Studies on chronic LF have shown proinflammatory cytokines such as TNF-γ, IL-6, soluble TNF receptor, IL-2 and endothelin-1, are elevated during manifestation of the disease (Das et al., 1996; el-Sharkawy et al., 2001; Satapathy et al., 2006). It has also been suggested that the infiltration of circulating peripheral blood mononuclear cells (PBMCs) across the lymphatic endothelia lining and that of other tissues (where filaria worms are sited) results in immune-mediated inflammations (Freedman, 1998). Recently, Nathan et al. (2017) reported that PBMCs from filaria lymphoedema individuals are defective and their lymph vasculogenic function is irregular compared to PBMCs from endemic normal.

Furthermore, mouse models for human filaria parasites are restricted since none of the parasites can complete their life cycle in mice. Nonetheless, surgical implantation of adult *B. malayi* into peritoneal cavity of mice has revealed the immunosuppressive capacity of alternatively activated macrophages (AAM) and $Foxp3^+$ Tregs (Allen et al., 1996; Babu et al., 2009) in human filariasis. However, the establishment of *L. sigmodontis* in its natural host, cotton rats and *B. pahangi* in gerbils, has paved way for immunopathological studies of filariasis. Recently, Karadjian et al. (2017) showed that pulmonary phase of migration of *L. sigmodontis* L3 was associated with lung damage which was identified with granulomas and haemorrhage and this has been attributed to the damage to the endothelium and parenchyma of the pulmonary cavity while crossing to the pleural cavity. It has also been revealed from animal studies that neutrophils are critical elements in host

innate protective response against murine filariae (*L. sigmodontis*) L3 at the early phase of infection, before the larvae reach their site of maturation (Pionnier et al., 2016).

2.7 CO-INFECTION OF FILARIA AND MALARIA PARASITES

2.7.1 Co-infection of filaria and malaria parasites in human

Co-infection of malaria and filaria parasites has long been studied in humans (Chadee et al., 2003; Ghosh & Yadav, 1995; Muturi et al., 2006; Prasad et al., 1990; Ravindran et al., 1998) and the interactions that occur between malaria and filaria parasites within a single host has been least studied in humans (Muturi et al., 2008).

The prevalence of co-infection of filaria and malaria parasites in humans, is often low most times. It has been suggested that co-infection will most likely occur where the prevalence of both diseases is high (Muturi et al., 2008). Some studies have reported that mosquitoes can ingest mf from ultralow microfilaremic individuals (Lowrie Jr et al., 1989; Subramanian et al., 1998). Previous reports on prevalence of co-infection of filariasis and malaria in India was less than 1% (Ghosh & Yadav, 1995; Prasad et al., 1990; Ravindran et al., 1998); and in Guyana, it was between 0.4 – 3.3% (though about 83% of the subjects were Africans) (Chadee et al., 2003). However, much higher prevalence has been reported in Africa. For instance, prevalence of 11.1% co-infection of *W. bancrofti* and *P. falciparum* was reported among school children in Tanzania, and it was noteworthy that single infection of malaria and filariasis reported in that study was 75.3% and 62.9% respectively (Mboera et al., 2011).

Moreover, very few studies have been conducted on the interactions that exist between filaria and malaria parasites during course of co-infection in humans. A prospective study on co-infection of filariasis and malaria with six months follow up amongst children and adults in Mali, has shown that existing filaria infection protects against severe malaria anaemia and serum level of inflammatory cytokines decreased, but no significant

difference on susceptibility to or severity of malaria (Dolo et al., 2012). Similarly, some studies conducted on immune responses to concomitant infection of filariasis and malaria amongst teenagers (ages 11 – 20 yrs) from Mali, showed that filaria infection alters the quality and magnitude of malaria-specific T-cells; modulates *P. falciparum* specific IL-2p 70/ IFN-γ secretion pathways which is IL-10 dependent; and also reduce Th1 and Th17 T cells (Metenou et al., 2012; Metenou et al., 2009; Metenou et al., 2011). In lieu of this, it is therefore imperative to conduct more research on co-infection of filariasis and malaria in animal models, in order to elucidate on the mechanisms and interactions that exist between these infections.

2.7.2 Co-infection of filaria and malaria parasites in murine models

Previously, inoculation of mice with irradiated attenuated infective larvae of *B. pahangi* conferred protection against development of cerebral malaria when co-infected with *P. berghei* ANKA (PbA), although anaemia was aggravated and all mice later died (Yan et al., 1997). It has also been reported that there was no difference in disease outcome in co-infection of *Heligmosoides polygyrus* and PbA in mice (de Souza & Helmby, 2008; Tetsutani et al., 2008), while reports had shown that infection of *L. sigmodontis* conferred protection against severity of PbA infection in mice (Ruiz et al., 2009; Specht et al., 2010).

Conversely, the efficacy of vaccination of Balb/c mice with *P. berghei* circumsporozoite protein was diminished in *L. sigmodontis* infected mice (Kolbaum et al., 2012). Similarly, concurrent co-infection of *H. polygyrus* and *P. chabaudi* AS reduced the protective efficacy of erythrocyte-stage malaria vaccine in immunized C57BL/6 mice (Su et al., 2006). However, report by Graham et al. (2005) has shown that in co-infection of *L. sigmodontis* and *P. chabaudi* AS, amicrofilaremic (mf-) mice had more severe anaemia and significant elevated IFN-γ from polyclonal splenic cells, whereas in

microfilaremic (mf+) mice, the induced immune regulation was less pronounced. More so, concurrent co-infection of *H. polygyrus* and *P. yoelii* 17XNL in BALB/c mice resulted in exacerbation of the malaria infection and it was characterized by suppression of IFN-γ and elevated IL-4 (Noland et al., 2008).

Therefore, it is very clear that the outcomes of co-infection of filaria nematodes and malaria parasite in murine models are influenced by genetics of the host, stage and type of filaria worms, stage and strains of *Plasmodium*, and sometimes the results are conflicting (Table 2.1). Until now, there are relatively scarce reports on co-infection of *B. pahangi* and PbA in gerbil (*Meriones unguiculatus*). Therefore, in this study, gerbil was used to establish as an experimental model for co-infection of *B. pahangi* and PbA, in order to evaluate the interactions that interplay between the 2 parasites in a single host.

Table 2.1: Outcome of nematode co-infection with *Plasmodium* in laboratory murine models

No.	Source	Filarial spp.	*Plasmodium spp.*	Host Strain	Outcome
1	Karadjian et al. (2014)	*L. sigmodontis*	*P. yoelii* 17XNL	BALB/C	Protection
			P. yoelii 17XNL	BALB/C	Aggravation
2	Kolbaum et al. (2012)	*L. sigmodontis*	*P. berghei*	BALB/C	Alter vaccine efficacy
3	Specht et al. (2010)	*L. sigmodontis*	*P. berghei* ANKA	C57BL/6	Protection
4	Ruiz et al. (2009)	*L. sigmodontis*	*P. berghei* ANKA (liver stage)	BALB/C	Protection
5	Tetsutani et al. (2009)	*H. polygyrus*	*P. yoelii* 17X NL	C57BL/6	Aggravation
6	Segura et al. (2009)	*H. polygyrus*	*P. chabaudi* AS	C57BL/6	Protection
7	Helmby (2009)	*H. polygyrus*	*P. chabaudi* AS	C57BL/6	Aggravation
8	de Souza and Helmby (2008)	*H. polygyrus*	*P. berghei* ANKA	C57BL/6; BALB/C	No difference
9	Tetsutani et al. (2008)	*H. polygyrus*	*P. berghei* ANKA	C57BL/6	No difference
10	Noland et al. (2008)	*H. polygyrus*	*P. yoelii* 17X NL	BALB/C	Aggravation
11	Su et al. (2006)	*H. polygyrus*	*P. chabaudi* AS	C57BL/6	Suppresses malaria vaccin efficacy
12	Su et al. (2005)	*H. polygyrus*	*P. chabaudi* AS	C57BL/6	Aggravation
13	Graham et al. (2005)	*L. sigmodontis*	*P. chabaudi* AS	BALB/C	Aggravation
14	Yan et al. (1997)	*B. pahangi* (irradiated attenuated larvae)	*P. berghei*	CBA/J	Protection

2.8 ELIMINATION OF LYMPHATIC FILARIASIS AND MALARIA

Lymphatic filariasis (LF) is among the infectious diseases targeted for global elimination, due to non-availability of animal reservoir host for *W. bancrofti* and only restricted foci for cat or dog as reservoir host for *B. malayi* (Ambily et al., 2011; Wongkamchai et al., 2014), and hence, calls for effective strategies to halt transmission (Cano et al., 2014). The strategy adopted to eliminate LF includes the establishment of the global programme to eliminate lymphatic filariasis (GPELF) in the year 2000, which has been advocating and recommending mass drug administration (MDA) of either albendazole plus diethylcarbamazine citrate, or albendazole plus ivermectin in areas co-endemic for onchocerciasis or twice yearly of albendazole only in areas co-endemic for loiasis (Kelly-Hope et al., 2014; WHO, 2016a). The MDA treatments have limited impact on the adult worms and hence, it is aimed at reducing the density of circulating mf in the blood of infected individuals and eventually, impedes transmission. The programme has been successful by annual repeated rounds of MDA for at least 5 years, as recommended by WHO (2008). Studies have shown that economic benefits of GPELF include prevented infection, halted disease progression, ancillary treatment of co-infections, over 175 million averted disability-adjusted life years (DALYs) in span of 15 years and over $2.2 billion saved by health systems of endemic countries (Chu et al., 2010; de Vlas et al., 2016; Turner et al., 2016).

In addition, more efforts have been intensified in mapping LF with the use of simple and rapid diagnostic tests (RDT), immune-chromatographic test (ICT) such as antigen-based test for *W. bancrofti* and antibody-based test for *Brugia* spp. (Cano et al., 2014). The advents of these tools have tremendously helped to eliminate the stress in collection of blood at night and also time consuming in preparation and examination of stained blood slides (Melrose & Rahmah, 2006; Rahmah et al., 2001; Supali et al., 2004).

Recently, the efforts to eliminate malaria has been re-energized by the launching of WHO global technical strategy (GTS) for malaria 2016 – 2030, roll back malaria advocacy plan, action and investment to defeat malaria (AIM), and sustainable development goals (SDGs) (WHO, 2017). The GTS and AIM are embedded in the SDGs in order to end disease such as acquired immune deficiency syndrome (AIDS), malaria, tuberculosis and a host of neglected tropical diseases by year 2030 (WHO, 2017). In the activities aligned to control malaria, vector control has been the main headway to achieve this, and it includes insecticide treated nets (ITNs) and indoor residual spraying (IRS). The use of ITNs has been successful in reducing over half of malaria incidence rate and malaria mortality in under 5-year-old children in sub-Saharan Africa (Eisele et al., 2010; Lengeler, 2004; WHO, 2017). In addition, increase in the distribution and coverage of malaria RDTs, which have led to considerable improvements in the accuracy of diagnosis, and advocacy on effective antimalarial drugs have all contributed to the achievement so far. Moreover, there are challenges to elimination of malaria which include the fast spreading antimalarial drug resistance (O'Brien et al., 2011; White, 2004), insecticide resistance (Hemingway et al., 2004; Liu, 2015; Liu et al., 2006; Ranson et al., 2011), and the false-negative diagnosis by histidine-rich protein 2 (HRP-2) or HRP-3 RDTs recommended by WHO (Bell et al., 2005; Koita et al., 2012; Kumar et al., 2013). The challenges on anti-malaria drug resistance have long been identified with chloroquine (Babiker et al., 2001; Krogstad et al., 1987), quinine (Sidhu et al., 2005; Zalis et al., 1998), sulphadoxine pyrimethamine (Plowe et al., 1997; Sibley et al., 2001), and more recently, artemisinin resistance (Ashley et al., 2014; Hemming-Schroeder et al., 2018; Kobasa et al., 2018).

Moreover, recent findings have shown that the implementation of MDA in urban settings of the endemic areas, is no longer obtainable and this poses threat to the required 65% MDA coverage for elimination of LF (Koudou et al., 2018; Pam et al., 2017). There

have been calls for thorough investigation on the role/effect of long lasting insecticidal nets (LLINs)/bed nets in the control of LF where MDA is absent ant its double effects where LF is co-endemic with malaria (Molyneux et al., 2014). However, it has been suggested that the vector control and management adopted in the elimination of malaria, will significantly block the transmission of LF, since both parasites are mainly transmitted by *Anopheles* female mosquitoes in endemic communities (Bockarie et al., 2009; Kelly-Hope et al., 2013; Manga, 2002). Previous reports have shown that hypo-responsiveness to helminths may actually alter the immune response to a potential malaria vaccine (Hartgers & Yazdanbakhsh, 2006; Nacher, 2001). In animal studies, nematodes such as *L. sigmodontis* and *H. polygyrus* have been shown to alter the efficacy of malaria vaccines (Kolbaum et al., 2012; Su et al., 2006). Therefore, there is a need to take cognizance of co-infection of helminths and malaria in the ongoing elimination of both parasitic infections.

CHAPTER 3: PATHOGENESIS OF *PLASMODIUM BERGHEI* ANKA INFECTION IN THE GERBIL *(MERIONES UNGUICULATUS)* AS AN EXPERIMENTAL MODEL FOR SEVERE MALARIA

3.1 INTRODUCTION

According to the World Health Organization (WHO), an estimated 214 million new cases of malaria (against 237 million cases in year 2010) and 438,000 deaths were recorded in the year 2015 (WHO, 2016b). In order to alleviate this burden, there is still a need to better understand the underlying processes that result in severe disease outcome and mortality. One of the ways this can be achieved is by exploring different experimental models for malaria.

Similar to human malaria infections, the clinical outcomes of rodent malaria vary in virulence depending on the species of *Plasmodium* and species or strains of the rodent hosts (Sanni et al., 2002; Stephens et al., 2012). It has been suggested that the clonal composition of the *Plasmodium* parasite has an effect on the disease outcome and also, the parasite virulence can be regulated by the host genetic background and the dynamics interplay between the clones and their hosts (Amani et al., 1998).

Severe malaria anaemia (SMA) is a common occurrence in malaria endemic communities and is considered to be responsible for high morbidity and mortality in young children and pregnant women (Greenwood, 1997; Lamikanra et al., 2007). Previously, the clinical features and pathogenesis of severe malaria were attributed to either severe anaemia due to destruction of red blood cells (RBC) or cerebral malaria (CM), which is caused by obstruction of small vessels of the brain by sequestered parasites (Mackintosh et al., 2004). However, the host has evolved a mechanism in controlling the degree of RBC destruction, which is beneficial when the host effectively

remove infected RBCs, while it is detrimental when the host destroys both infected and uninfected RBCs.

The ANKA strain of *P. berghei* (PbA) has long been used as a model for experimental cerebral malaria (ECM) due to its high degree of reproducibility and the development of histopathological and neurological symptoms similar to human cerebral malaria (CM) (de Miranda et al., 2011; Lou et al., 2001). A previous study by Bopp et al. (2010) has shown that different mouse strains infected with PbA are either susceptible or resistant to ECM to varying degrees.

Early secretion of pro-inflammatory T-helper 1 (Th 1) cytokines is important in successful resolution of malaria infection through killing of parasites by macrophages, thus preventing immune-mediated damage (Julius et al., 2013). Although pro-inflammatory cytokines are crucial in the clearance of *Plasmodium* parasites, their overproduction has been associated with severe outcome that follows *Plasmodium* infection (Artavanis-Tsakonas et al., 2003; Wu et al., 2014). On the other hand, the inability of the host to mount an effective pro-inflammatory response, may instead lead to unhindered parasite replication, hence aiding severe immunopathology (Couper et al., 2008). These observations suggest that the balance between pro-inflammatory and regulatory immune responses during malaria infection is an important factor in determining the disease outcome.

Gerbils have been used in various areas of biomedical research, such as stroke, behaviour, parasitology, epilepsy, radiobiology, hearing and infectious disease research (An et al., 2003; Casals et al., 2011; de La Rochefoucauld & Olson, 2010; Hrapkiewicz et al., 2013). More importantly, gerbils have been established as a good experimental model for filaria nematodes (Porthouse et al., 2006; Rao & Klei, 2006), *Helicobacter pylori*-induced gastritis (Bleich et al., 2005; Yamaoka et al., 2005), and inflammatory

bowel disease (Bleich et al., 2010). Although mice and rats have been used extensively for experimental malaria studies, there are still doubts concerning the extrapolation of findings to severe malaria in humans (Lou et al., 2001). As a result, studying a relatively unexplored experimental model subjected to severe malaria will enhance understanding of the disease. Moreover, studies involving *P. berghei* in gerbils are not recent (Sergent & Poncet, 1951; Sergent & Poncet, 1956; Wellde et al., 1966), with the most recent study conducted over 4 decades ago (Weiss, 1976).

The pathology of PbA infection in mice has been associated with accumulation of infected RBCs in the brain, but it is not clear whether cyto-adherence of PbA occurs in the microvasculature of the brain (Craig et al., 2012). Baptista et al. (2010) have demonstrated that parasitized RBCs together with $CD8^+$ T cells play a crucial role in the onset of neuropathology in ECM. However, previous studies on PbA-mice models have also shown the presence and accumulation of iRBCs in various organs such as the brain, heart, liver, spleen, lungs and kidneys (Claser et al., 2011; Deroost et al., 2012; Deroost et al., 2014; Deroost et al., 2013). In addition, different methods and approaches have previously been used to identify and determine the accumulation of *Plasmodium* parasites in different organs (Deroost et al., 2012; Franke-Fayard et al., 2010; Frevert et al., 2014; Genrich et al., 2007).

Therefore, the objective of this study is to establish PbA infection in gerbils and to evaluate the clinical outcomes.

3.2 MATERIALS AND METHODS

3.2.1 Gerbils

Mongolian gerbils (*Meriones unguiculatus*) were purchased from Charles River (Massachusetts, USA) at approximately 4 wks old. They were maintained and allowed to breed at the animal facility of the Faculty of Medicine, University of Malaya. Gerbils

were maintained in individually ventilated cages and supplied with sterilized food and water *ad libitum*. The animals were housed according to their age and sex in accordance with institutional guidelines for animal care (2014/PARA/R/JOQ). All animals were handled humanely to minimize pain.

3.2.2 Ethics approval

The protocol was approved by the Faculty of Medicine Institutional Animal Care and Use Committee (FOM IACUC), University of Malaya, Malaysia (2014/PARA/R/JOQ) (Appendix 1).

3.2.3 Adaptation of *Plasmodium berghei* ANKA and infection

Plasmodium berghei ANKA (MRA-311) was obtained from Malaria Research and Reference Reagent Resource Center (MR4, USA), contained in 0.5 mL blood vial. About 0.2 mL each was injected intraperitoneally into 2 naïve gerbils (age and sex matched). One of the gerbils developed parasitaemia after 5 days post infection (pi), while the other gerbil was observed for over 15 days without any signs or symptoms of the infection. The infected gerbil was euthanized at day 10 pi after draining the blood through cardiac puncture under anesthesia, although the parasitaemia at this point was less than 15%. The harvested infected red blood cells (iRBCs) were then cryopreserved as stocks in liquid nitrogen (for long term storage) and -80 ^{o}C freezer (for short term storage and routine use). Parasites were stored in liquid nitrogen or -80 ^{o}C freezer with 10% glycerol in Alsever's solution (2.33 g of glucose, 1 g of sodium citrate and 0.52 g of sodium chloride in 100 mL double distilled water).

To start the experiments, frozen PbA parasitized red blood cells (iRBCs) were thawed at 37°C for 3-5 mins, and 0.2 mL was injected into an uninfected gerbil to initiate infection (Figure 3.1). Blood was collected by cardiac puncture from the donor gerbil on

days 5-7 post-infection, and diluted with phosphate buffer saline (PBS, pH 7.4), then counted using counting chambers, before re-introduction into new gerbils. Control animals were given only phosphate buffer saline (PBS, pH 7.4).

3.2.4 Parasitaemia, survival rates and disease assessment

Gerbils were inoculated intraperitoneally with different quantities (10^7, 10^6, 10^5, 10^4, 10^3, 10^2) of PbA to determine their susceptibility to PbA infections. Parasitaemias were monitored every 48 hrs by thin blood smears prepared from tail pricks. These were then fixed in methanol and stained with 3% Giemsa (Sigma, USA) solution for 45 mins and allowed to dry before examining under a light microscope. Parasitaemias were quantified as the percentage of iRBCs in at least 5 microscopic fields, each containing approximately 200-250 RBCs. To evaluate their survival rate, gerbils were monitored daily for a period of 30 days.

The body weight of gerbils was measured using an electronic balance (A&D, Japan), while body temperature in the animals was measured with a thermometer (Rossmax TG380, Switzerland) by placing the thermometer probe 0.5-1 cm into the mouth. Haemoglobin (Hb) concentrations were determined by Hemocue AB (Angelholm, Sweden). Briefly, 5-10 µL of blood from a tail prick was pipetted into the cuvette, which was inserted into Hemocue and readings were taken. Total red blood cell count was determined using a haemocytometer (Marienfeld, Germany) under a light microscope. This was done by collecting 5-10 µL of tail pricked blood into heparinized haematocrit-capillary tubes (Hirschmann, Germany). It was then diluted with phosphate buffered saline (PBS), and stained with trypan's blue (Sigma, USA). Gerbils were assessed daily (every 6 hours) for clinical symptoms such as ruffled hair, hunchback, coma, convulsion, paralysis and wobbly gait.

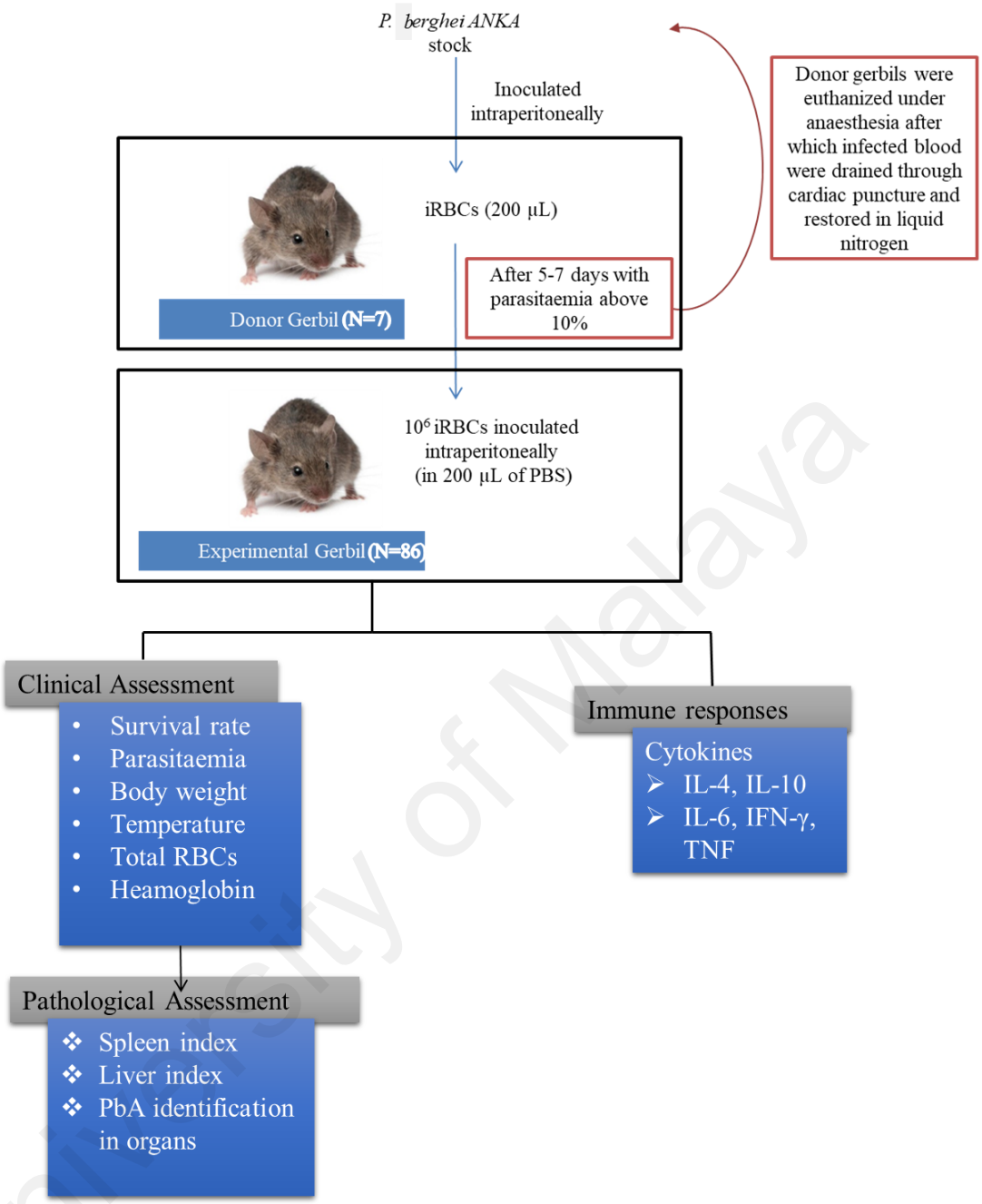


Figure 3.1: Establishment and assessments of *Plasmodium berghei* ANKA infection in gerbils

3.2.5 RNA extraction and cDNA preparation

The animals were first anaesthetized with ketamine (Troy Laboratories, Australia) and xylazine (Santa Cruz Animal Health, USA) intraperitoneally at the dose of 50 mg/kg and 2 mg/kg, respectively (Hrapkiewicz et al., 2013). Blood was then drained from the gerbil through cardiac-puncture into EDTA tubes (BD, USA) on ice. Total RNA was extracted from the blood using a GF-1 Blood Total RNA Extraction kit (Vivantis, Malaysia). Simultaneously, about 50-100 mg of the brain and spleen were surgically removed and the tissues snapped frozen in liquid nitrogen before extracting their total RNA with a Pure link RNA Mini kit (Life technologies, USA), in accordance with the manufacturer's instructions. The quantity and quality of the RNA were then assessed with the NanoDrop 2000 spectrophotometers (Thermo Fisher Scientific, USA). Total RNA of 2 µg was then converted to cDNA with a Super Script IV first-strand synthesis kit (Thermo Fisher Scientific, USA). A single 20 µL reverse transcription reaction was prepared in the following proportion: 50 ng/µL random hexamers (1 µL); 10 mM deoxynucleotide (dNTP) (1 µL); 2 µg RNA (up to 11 µL); diethylprocarbonate (DEPC)-treated water (up to 13 µL); 5× Super Script IV buffer (4 µL); 100 mM dithiothreitol (DTT) (1 µL); ribonuclease inhibitor (1 µL); and Super Script IV reverse transcriptase (200 U/µL) (1 µL). The cDNA was then used as a template for the RT-PCR.

3.2.6 Primers and probes

Gene-specific oligonucleotide primers and probes for gerbils IL-4 (GenBank L37779), IL-6, IL-10 (GenBank L37781), IFN-γ (Gen Bank L37782), TNF (GenBank AF171082.1) and GAPDH (GenBank AB040445.1), have been published previously (Yamaoka et al., 2005) and were incorporated in the study. Both the primers and probes for the Taqman RT-PCR assay used were from Applied Biosystems (Life technologies, USA), and these were further modified with the probes labelled reporter dye 6-

carboxyfluorescein (FAM) at 5' and quencher minor groove binder (MGB) at 3'. The list of primers and their sequences are given in Table 3.1.

3.2.7 Analysis of cytokines by real-time PCR

Taqman PCR reactions for cytokine mRNA and housekeeping GAPDH mRNA levels were performed using an Applied Biosystems StepOnePlus Real-Time PCR system (Life technologies, USA). Taqman reactions (20 µL) were performed in triplicate using Taqman Fast Advanced Master Mix (Life technologies, USA) according to the manufacturer's instructions. The qPCR was carried out with slight modification to the manufacturer's instructions as follows: incubation at 50°C for 2 min; polymerase activation at 95°C for 20 secs; and 40 cycles of PCR with denaturation at 95°C, 3 secs and annealing/extension at 60°C for 30 secs. Comparative standard curves were generated as a result, with the data being presented as the mean fold change of the cytokine mRNA relative to the level of GAPDH mRNA.

3.2.8 Histopathology

The spleen and liver of gerbils were assessed morphologically. The spleen index was calculated as the ratio of spleen wet weight (g) to body weight (g) x100 (Specht et al., 2010), whereas the liver index was calculated as the ratio of liver wet weight (g) to body weight (g) x100.

The carcasses of the gerbils subjected to clinical assessment and survival tests were preserved for post mortem. The brain, liver, spleen, lungs, kidneys and heart were removed and preserved in 10% buffered formalin. The tissues were processed using an automated tissue processor (Leica TP1020, USA) and then embedded in paraffin wax. About 3-5 sections (4 µm) were randomly cut for both haematoxylin and eosin (H and E) staining and *in situ* hybridization.

3.2.9 *Plasmodium* genus probe

An oligonucleotide probe labelled with digoxigenin-dUTP was designed to detect a part of the small subunit (18S) ribosomal RNA sequence of *Plasmodium* species that was available from the GenBank database. The probe sequence was 5'-GAAGTTTAAGGCAACAACAGGTCTGTGATGTCCTTAGATGAACTAGGCTGC ACGCG-TGCTACA- 3', with GenBank M19173.1 and U07368.1 (Appendix B). The probe was a product of *Plasmodium* RNA sequence characterization project yet to be published (courtesy: Dr. Lau LY and Dr. Ong KC). The sequence was submitted to the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov/blast.cgi) to check for its specificity. Although, the probe was originally designed for human malaria species which was confirmed upon BLAST with 100% identity with all human malarial species, it however showed 97% identity with most rodent malaria species including PbA, *P. yoelii* and *P. chaubaudi*.

3.2.10 *In situ* hybridization

The paraffin wax embedded tissues were sectioned (4 µm thick) and placed on salinized frosted slides. The tissues were dewaxed in xylene and rehydrated in series of graded alcohol (100% and 95%) and distilled water. Pigments were removed from the tissues with 10% ammonium (in 70% alcohol) for 10 mins. The tissues were then washed in distilled water and double distilled water, followed by antigen retrieval in citrate buffer on cooker for 10 mins. The tissues were then allowed to cool before being treated with 0.1% pepsin for 30mins at 37^{o}C. This was followed by 2 times washing in phosphate buffered saline (PBS).

The tissues were covered with 50 µl hybridization buffer which contained: 15 µl of standard sodium citrate (SSC), 5 µl of 50x Denhardt's solution, 0.5 µl of Salmon Sperm

DNA (Invitrogen, USA), 4.5 µl of double distilled water, 25 µl of dextran sulphate (10% w/v) and 1 µl *Plasmodium* genus probe.

The slides were then incubated at 95ºC for 10 mins, then overnight at 42ºC. On the second day, the slides were washed in 6x SSC (2 times), 2x SSC and Tris- NaCl buffer. The slides were then incubated with 0.5% blocking reagent (in Tris-NaCl) for 30 mins at room temperature (RT), followed by incubation with anti-digoxigenin-AP Fab fragments (Roche, Switzerland) in Tris-NaCl (1:2000 dilution) at 4ºC overnight.

At day 3, the slides were washed in Tris-NaCl (x3), and incubated with liquid permanent red chromogen (Dako, USA) (1:100 dilution) for 2 hrs at RT. The slides were then washed with tap water for 10 mins, followed by counter staining with Mayer's haematoxylin (Sigma, USA) and mounted with Faramount aqueous mounting medium (Dako, USA).

3.2.11 Statistical analysis

All data were analysed using GraphPad prism 6. The distribution of the data was assessed by a Kolmogorov-Smirnov test for normality testing. Data that followed normal distribution were analysed by one-way ANOVA with Tukey's multiple comparison post-hoc tests in cases of significant differences. All results are expressed as mean $\pm$ S.E.M (Standard Error of Mean) and considered statistically significant when $p < 0.05$.

Table 3.1. Primers and Probes used in this study

Gene	Sequence
GAPDH	Forward primer: 5' -CAAGCCCATCACCATCTTCCA- 3' Reverse primer: 5' -CGGTGGACTCCACAACATACTC- 3' Probe: 5' -FAM-CCGCCAACATCAAATG-MGB- 3'
IL-4	Forward primer: 5’ -CAGGGTGCTCCGCAAATTT- 3’ Reverse primer: 5’ -GACCCCGGAGTTGTTCTTCA- 3’ Probe: 5’ -FAM-ACTTCCCACGAGAGGTG-MGB- 3’
IL-6	Forward primer: 5’ -AGGATCCAGGTCAAATAGTCTTTCC- 3’ Reverse primer: 5’ -TTCCGTCTGTGACTCCAGTTTCT- 3’ Probe: 5’ - FAM-CCCAACTTCCGAGGCG-MGB- 3’
IL-10	Forward primer: 5’ -CAAGGCAGCCTTGCAGAAG- 3’ Reverse primer: 5’ -TCCAGCCAGTAAGATTAGGCAATA- 3’ Probe: 5’ - FAM-CTCCATCATGCCCAGCT-MGB- 3’
IFN-γ	Forward primer: 5’ -TTGGGCCCTCTGACTTCGT- 3’ Reverse primer: 5’ -CAGTGTGTAGCGTTCATGGTCTCT- 3’ Probe: 5’ -FAM-CCGGACTTGCCCTGC-MGB- 3’
TNF	Forward primer: 5’ -CACTCAGGTCCTCTTCTCAGAAC- 3’ Reverse primer: 5’ -TGGTGGTTGGGTACGACATG- 3’ Probe: 5’ -FAM-CCAGCGACAAGCCTG-MGB- 3’
	FAM: 6-carboxyfluorescein; MGB: Minor Groove Binder

3.3 RESULTS

3.3.1 Susceptibility of gerbils to PbA infection

The adaptation of PbA to gerbil showed that 100% infection could not be recorded until third passage and mortality of gerbil was observed from second passage. However, 100% infection and mortality were observed after the tenth passage (Table 3.2).

Different quantities (10^7, 10^6, 10^5, 10^4, 10^3, 10^2) of PbA iRBCs were administered intraperitoneally (ip), to determine the susceptibility of gerbils to PbA infections. Gerbils showed high susceptibility to PbA infections with 100% mortality recorded at all concentrations of iRBCs tested within 30 days post-infection (pi), with the exception of 10^2 iRBCs, showing 80% mortality by day 27 pi (Figure 3.2a). However, the duration for which parasites were detected in gerbils (days post-infection) depended on the amount of PbA given. Overall, no later than day 5 pi, all gerbils were tested positive. The parasitaemia observed during the course of the experiment did not exceed 70% and there was no significant difference ($F_{(5, 42)} = 1.579$, $p = 0.170$) in the parasitaemia level irrespective of iRBC concentrations (Figure 3.2b).

Table 3.2: Adaptations of PbA to gerbil

No. of passages	No. of animals	Parasitaemia (%)				Mortality n (%)
		0	≤ 1	2-15	>30	
P1	2	1	-	1	-	0 (0)*****
P2	3	1	1	1	-	1 (33.3)^
P3-5	5	-	1	3	1	2 (40.0)^
P6-8	5	-	-	2	3	4 (80.0)
P9-10	10	-	1	1	8	9 (90.0)
P11-15	5	-	-	-	5	5 (100.0)

***** The positive gerbil was used for passage.
^ Moribund and euthanized.
All PbA iRBCs used for the study were from cryopreserved blood after the tenth passage.

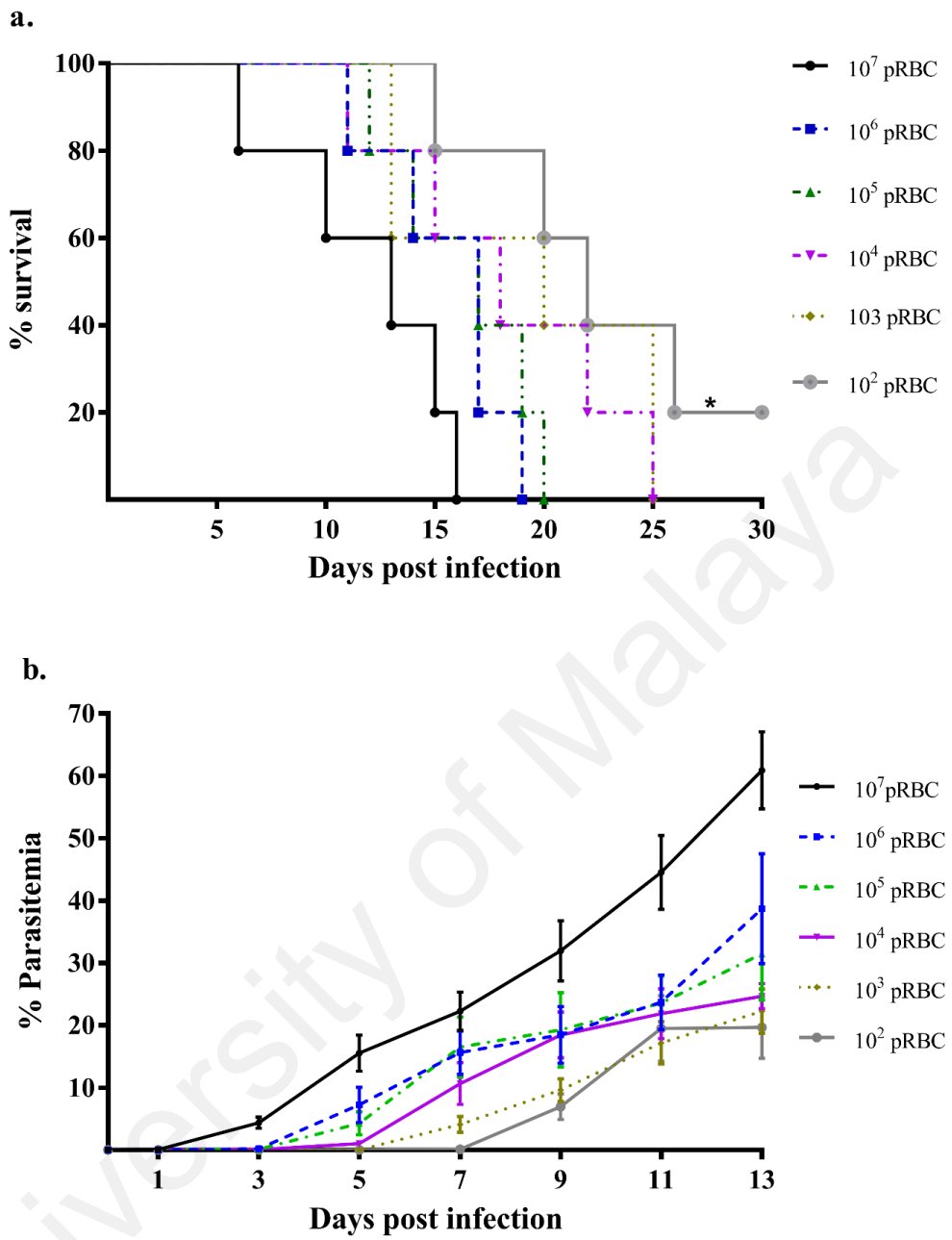


Figure 3.2: Susceptibility of gerbils to PbA infection.

Gerbils were infected intraperitoneally with different concentrations of PbA-parasitized red blood cells (pRBCs). Survival and parasitaemia were monitored daily and every 2 days (respectively). a. Survival of gerbils according to the concentration of iRBC. b. Parasitaemia level of PbA-infected gerbils according to the concentration of PbA, over 13 days post-infection. Lines represent mean ± S.E.M., N = 5 per group. A logrank (Mantel-cox) test was used to compare survival curves, * $p < 0.05$. One-way analysis of variance (ANOVA) was used to compare the differences in the level of parasitaemia between the groups, $p > 0.05$.

3.3.2 Pathogenesis of PbA infection in gerbils

A comparison between infected gerbils (1 $\times 10^6$ iRBCs in 200 µL of PBS) and the control group (200 µL of PBS only) was carried out to observe the possible pathological effects of PbA on the gerbil host. Both bodyweight and temperature changes during the course of infection were monitored. The body weight of PbA infected gerbils was observed to decline from day 3 pi and showed a significant difference ($F_{(7, 48)} = 8.328$, $p < 0.0001$) at days 9 and 11 pi, while the control animals gained weight steadily (Figure 3.3a). However, the decline in body temperature observed during the course of infection was more evident from day 5 pi, and was highly significant ($F_{(7, 48)} = 84.318$, $p < 0.0001$) (Figure 3.3b).

The gerbils were also assessed for the level of anaemia by quantifying the haemoglobin (Hb) level and total RBC counts during the course of PbA infection. It was observed that there was a significant ($F_{(7, 48)} = 180.220$, $p < 0.0001$) decline in Hb in the infected group compared to the control group over the time course (Figure 3.4a), and a similar pattern of decline was recorded in the total RBC count of the infected group (Figure 3.4b).

The common symptoms shared by the infected gerbils were ruffled hair and hunchback. However, none of the infected gerbils showed signs such as ataxia, convulsion and deviation of the head, except one gerbil that had partial paralysis (Table 3.3).

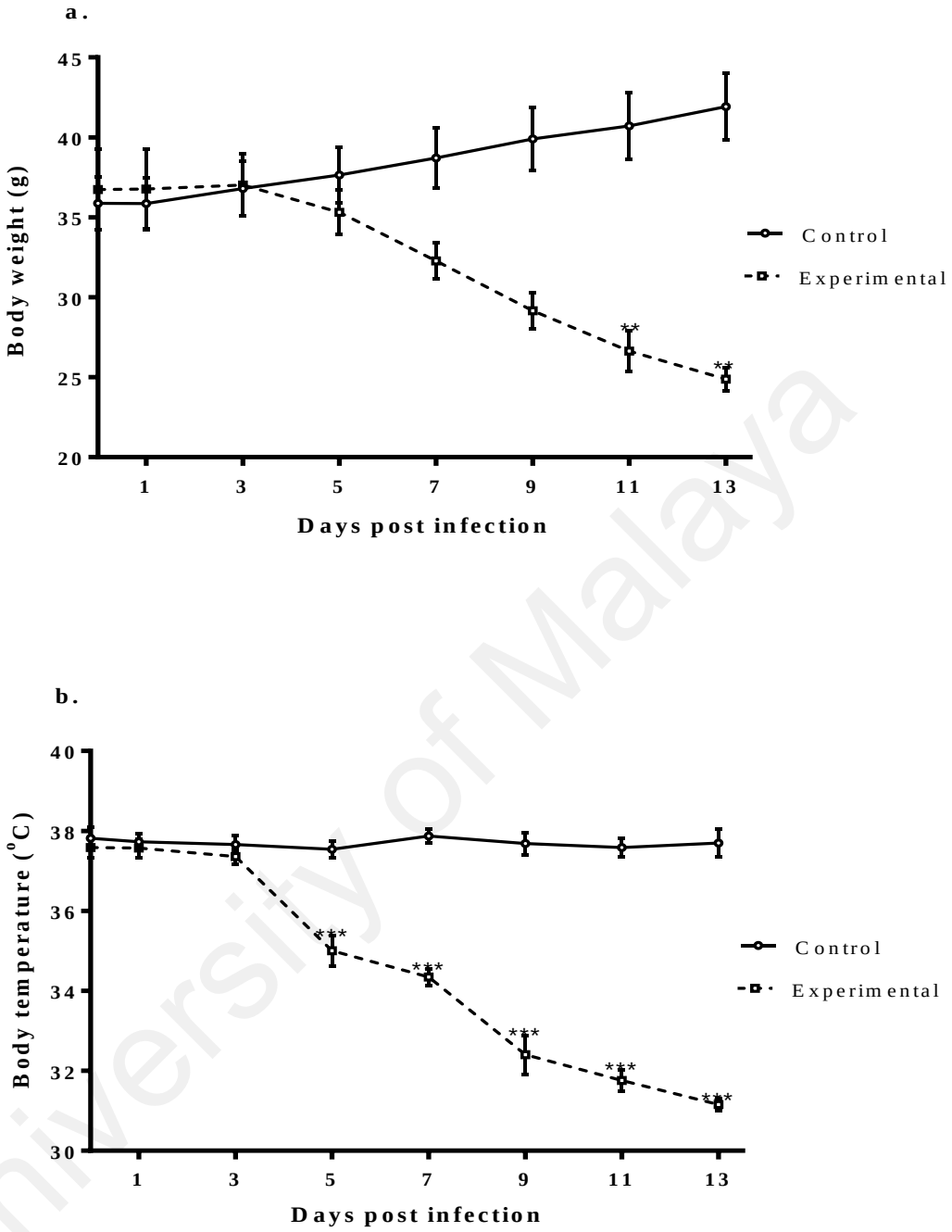


Figure 3.3: Body weight and body temperature during PbA infection.

a. Body weight of infected and control gerbil. b. Body temperature as measured in infected and control group. Lines represent mean ± S.E.M, while N = 7 per group. All data are representative of three independent experiments and compared by one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-hoc test for differences between groups (**$p < 0.005$; ***$p < 0.0001$). All data are representative of 3 different experiments.

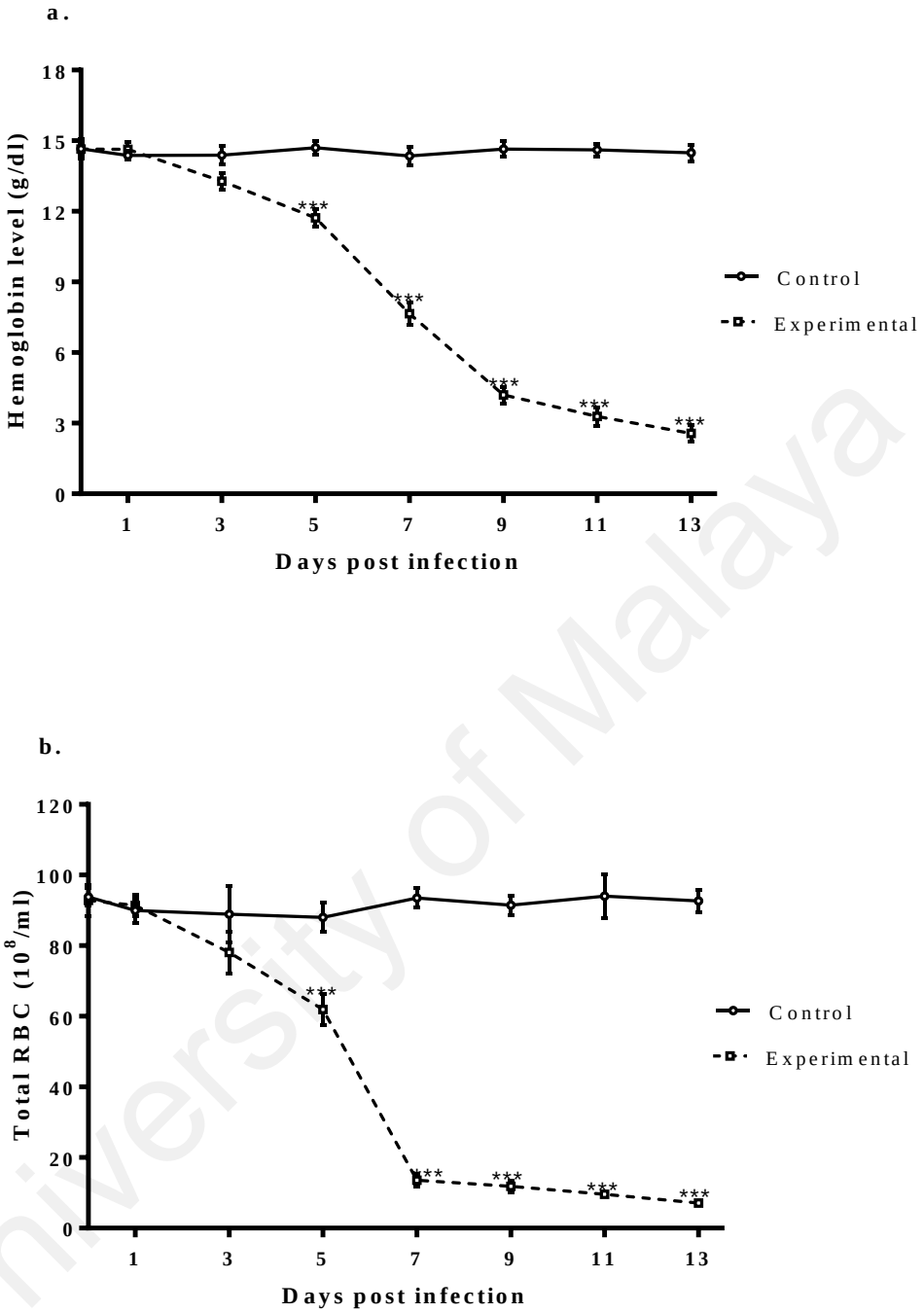


Figure 3.4: Haemoglobin and total RBCs during PbA infection.

a. Haemoglobin level as measured in infected and control group. b. Total RBCs as measured in infected and control group. Lines represent mean ± S.E.M, while N = 7 per group. All data are representative of three independent experiments and compared by one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-hoc test for differences between groups (***$p < 0.0001$). All data are representative of 3 different experiments.

Table 3.3: Clinical symptoms observed in infected gerbils

Symptoms	Quantities of iRBCs						Total
	10^7	10^6	10^5	10^4	10^3	10^2	N (%)
Ruffled hair	5	4	4	4	5	3	25 (83.3)
Hunchback	2	3	4	3	4	4	20 (66.7)
Ataxia	0	0	0	0	0	0	0 (0.0)
Convulsion	0	0	0	0	0	0	0 (0.0)
Wobbly gait	1	2	3	2	1	2	11 (36.7)
Deviation of the head	0	0	0	0	0	0	0 (0.0)
Paralysis	0	0	0	1	0	0	1 (3.3)*

Gerbils were observed daily to monitor their clinical symptoms. Five gerbils were assessed in each group, N = 30. * The gerbil showed partial paralysis of the hind limbs briefly before death. iRBCs: infected red blood cells.

3.3.3 Cytokine response to PbA infection

Pro-inflammatory cytokines (such as IL-6, IFN-γ and TNF), anti-inflammatory cytokines (IL-4) and immunomodulatory cytokines (IL-10) were chosen to study the immune response of gerbils to PbA infection. The mRNA levels of these cytokines were quantified in the brain, spleen and blood of gerbils at various time points after intraperitoneal infection. Over all, IL-10, IL-6, IFN- γ and TNF were significantly increased in the brain and spleen, whereas in the blood, only IL-10 and IFN- γ were significantly elevated.

The IL-4 levels at days 3 and 5 post-infection (pi) in the spleen were more significantly elevated ($F_{(6, 28)} = 4.511$, $p = 0.003$) than at later time points, with a mean fold change of about 2.02 and 4.01, respectively. Although, there was a slight elevation of IL-4 in the brain at days 5 and 9 pi, it was not significant ($F_{(6, 28)} = 1.185$, $p = 0.343$) compared with other days pi. IL-4 was significantly ($F_{(6, 14)} = 8.807$, $p = 0.0004$) down-regulated throughout the time course in the blood, except day 5 pi which was at the same level as day 0 pi (Figure 3.5a).

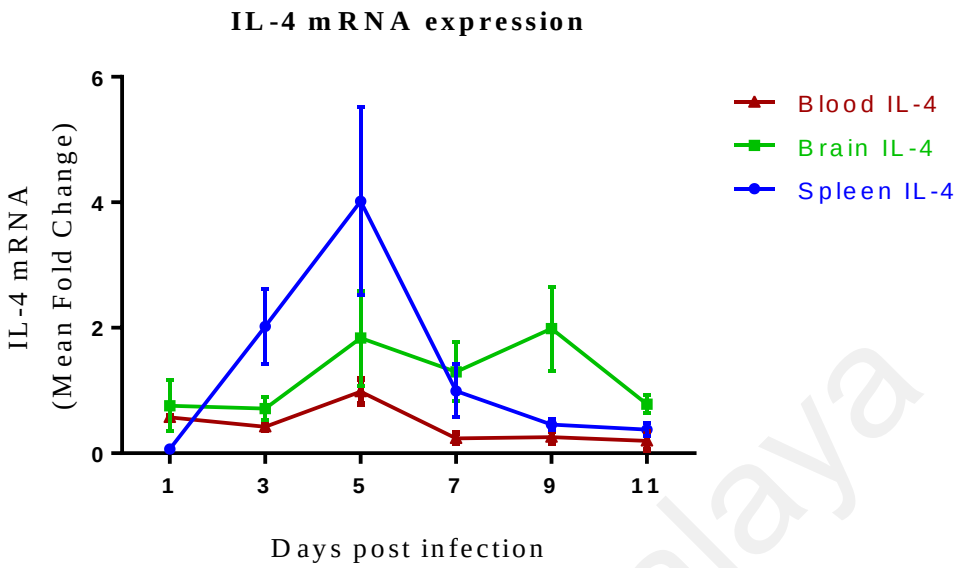
The profile of IL-10, as expressed in all the tissues, showed consistent dual peaks at days 5 and 7 pi which was significantly ($F_{(6, 28)} = 4.078$, $p = 0.005$ in spleen; $F_{(6, 28)} = 7.750$, $p < 0.0001$ in brain) expressed (Figures 3.5b). However, IL-10 was most significantly ($F_{(6, 14)} = 4.916$, $p = 0.007$) elevated at day 11 pi in the blood with a 6.3 fold change (Figure 3.5b).

The level of IL-6 mRNA expressed in the spleen and brain at day 5 pi was significantly ($F_{(6,28)} = 5.493$, $p = 0.001$; $F_{(6, 28)} = 3.019$, $p = 0.021$) up-regulated with 3.7 and 3.8 fold changes, respectively (Figure 3.6a). In the blood, IL-6 was only significantly ($F_{(6, 14)} = 2.904$, $p = 0.047$) expressed at day 7 pi (Figure 3.6a).

IFN in the spleen was significantly ($F_{(6, 28)}$ = 6.784, p = 0.0002) elevated only at day 5 pi, with a mean fold change of 4.5, then sharply lowered until day 11 pi. However, there was a significant ($F_{(6, 28)}$ = 3.910, p = 0.006; $F_{(6, 14)}$ = 13.273, p < 0.0001) increase in the expression of IFN both in the brain and blood from day 5 to day 11 pi (Figure 3.6b).

The expression of TNF mRNA was significantly ($F_{(6, 28)}$ = 2.924, p = 0.024) elevated from day 3 to day 9 pi in the spleen (Figure 3.6c). Although TNF mRNA was consistently high from day 3 to day 11 pi in the brain, the highest fold increase was observed on day 5 pi, with a fold change of 19.8, which was significantly ($F_{(6, 28)}$ = 7.145, p = 0.0001) higher than expression at other time points. There was no significant difference ($F_{(6, 14)}$ = 1.120, p = 0.399) in the expression of TNF in the blood throughout the time course (Figure 3.6c).

a.



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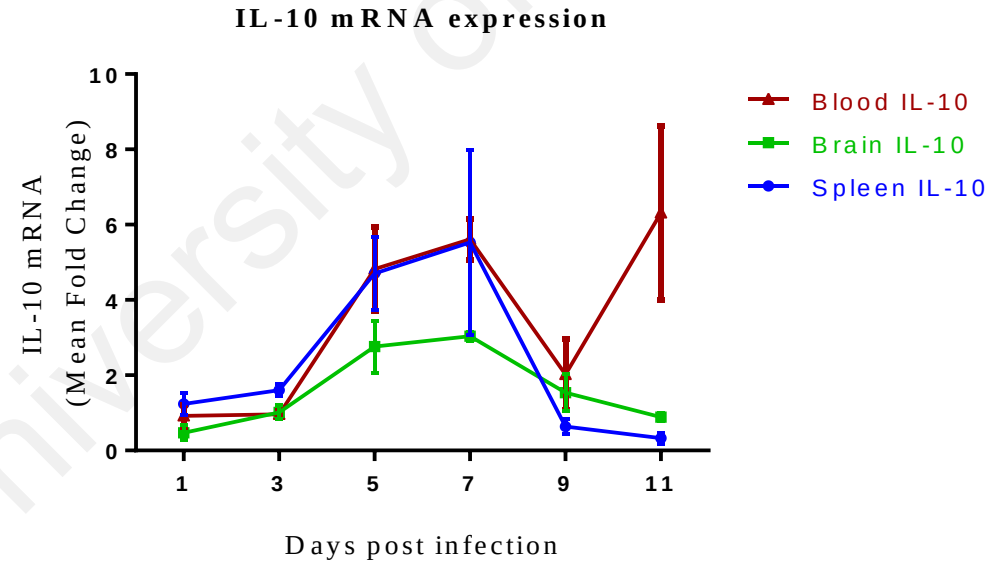

Figure 3.5: Quantitation of cytokine mRNA in the spleen, brain and blood.

Gerbils were euthanized under anaesthesia at days 1, 3, 5, 7, 9 and 11 after intraperitoneal inoculation with PbA. mRNA levels were measured by reverse transcription (RT)-PCR, and values were expressed as mean (± S.E.M, N = 5) fold changes compared with values from control, uninfected gerbils. a. IL-4 mRNA expression. b. IL-10 mRNA expression. All data are representative of 2 independent experiments and compared by one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-hoc tests for differences between groups.

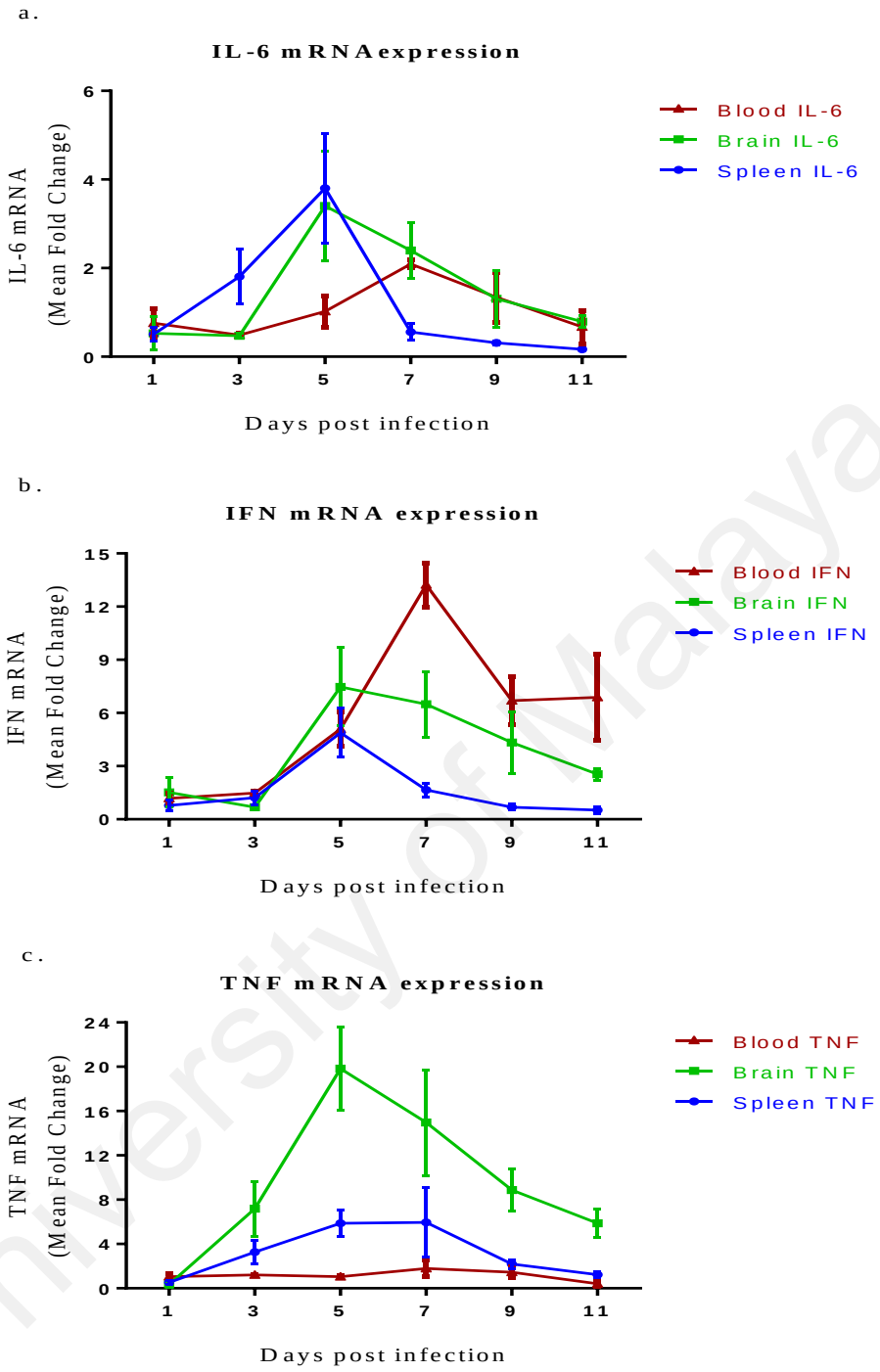

Figure 3.6: Quantitation of cytokine mRNA in the spleen, brain and blood.

Gerbils were euthanized under anaesthesia at days 1, 3, 5, 7, 9 and 11 after intraperitoneal inoculation with PbA. mRNA levels were measured by reverse transcription (RT)-PCR, and values were expressed as mean (± S.E.M, N = 5) fold changes compared with values from control, uninfected gerbils. a. IL-6 mRNA expression. b. IFN mRNA expression. c. TNF mRNA expression. All data are representative of 2 independent experiments and compared by one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-hoc tests for differences between groups.

3.3.4 Histopathology of *Plasmodium berghei* ANKA sequestration in the tissues

The physical features of organs such as the brain, lungs, heart, kidneys, spleen and liver were examined. Among the anomalies observed in the organs of infected gerbils, were the enlargement and discoloration (pigmentation) of the spleen and liver compared to those in the control group (Figure 3.7). Quantitatively, there was an increase in the spleen indices (ratios of spleen wet weight (g) versus body weight (g) x 100) and liver indices (ratios of liver wet weight (g) versus body weight (g) x 100) compared to that of the control group. The increments in the spleen and liver indices were significant ($F_{(7, 32)}$ = 224.205, $p < 0.0001$; $F_{(7, 32)}$ = 59.919, $p < 0.0001$, respectively) from day 7 pi (Figures 3.8a and 3.8b).

Formalin fixed paraffin embedded (FFPE) tissues from both PbA infected and uninfected gerbils were assessed on the following tissues: brain, kidneys, liver, lungs and spleen. The conventional method of haematoxylin and eosin (H and E) staining and *Plasmodium* genus DIG-labelled UTP probe *in situ* hybridization (ISH) on the tissues were compared. Results showed that PbA iRBCs were found in blood vessels of the tested organs and the ISH genus probe was sensitive and specific to the parasite (Figure 3.9).

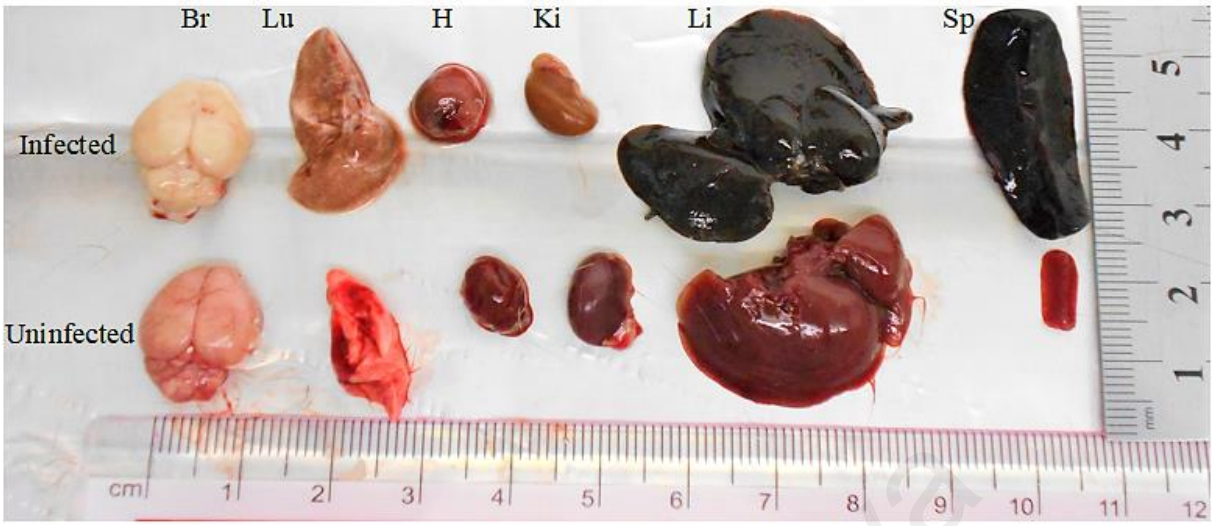

Figure 3.7: Morphology of organs harvested from gerbils at day 11 pi from infected and uninfected gerbils.

The organs are: Br: Brain; Lu: Lungs; H: Heart; Ki: Kidney; Li: Liver; and Sp: Spleen. The liver and spleen are pigmented and enlarged (hepatomegaly and splenomegaly, respectively), while the infected kidney, lungs and brain are pale.

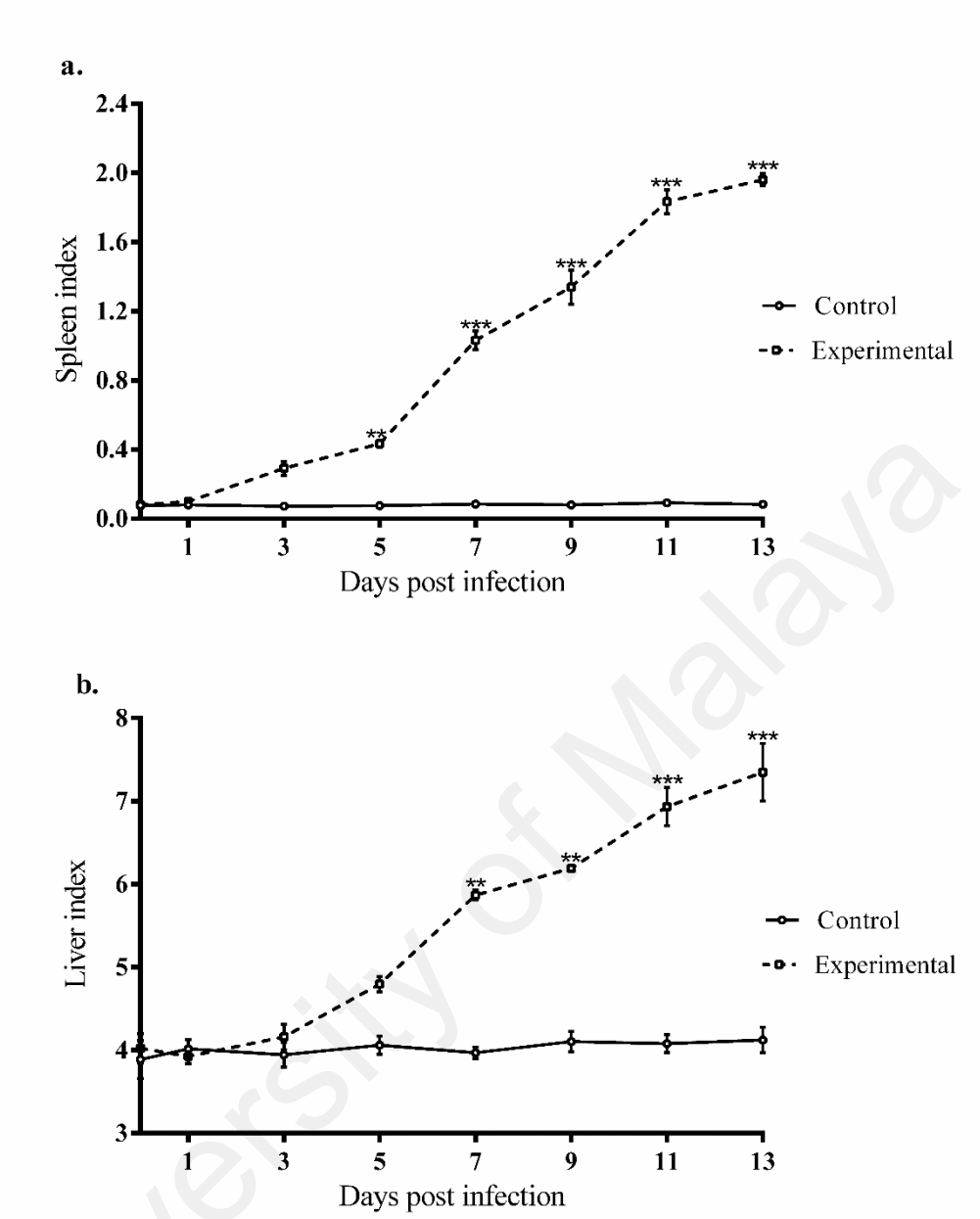

Figure 3.8: Spleen index and liver index

Measured as the ratio of the organ's wet weight (g) versus body weight (g) × 100) over the 13-day time course. a. spleen index. b. liver index. Lines represent mean ± S.E.M, while N = 5. All data were compared by one-way ANOVA with Tukey's multiple comparison post-hoc tests for differences between groups (**$p < 0.005$; ***$p < 0.0001$).

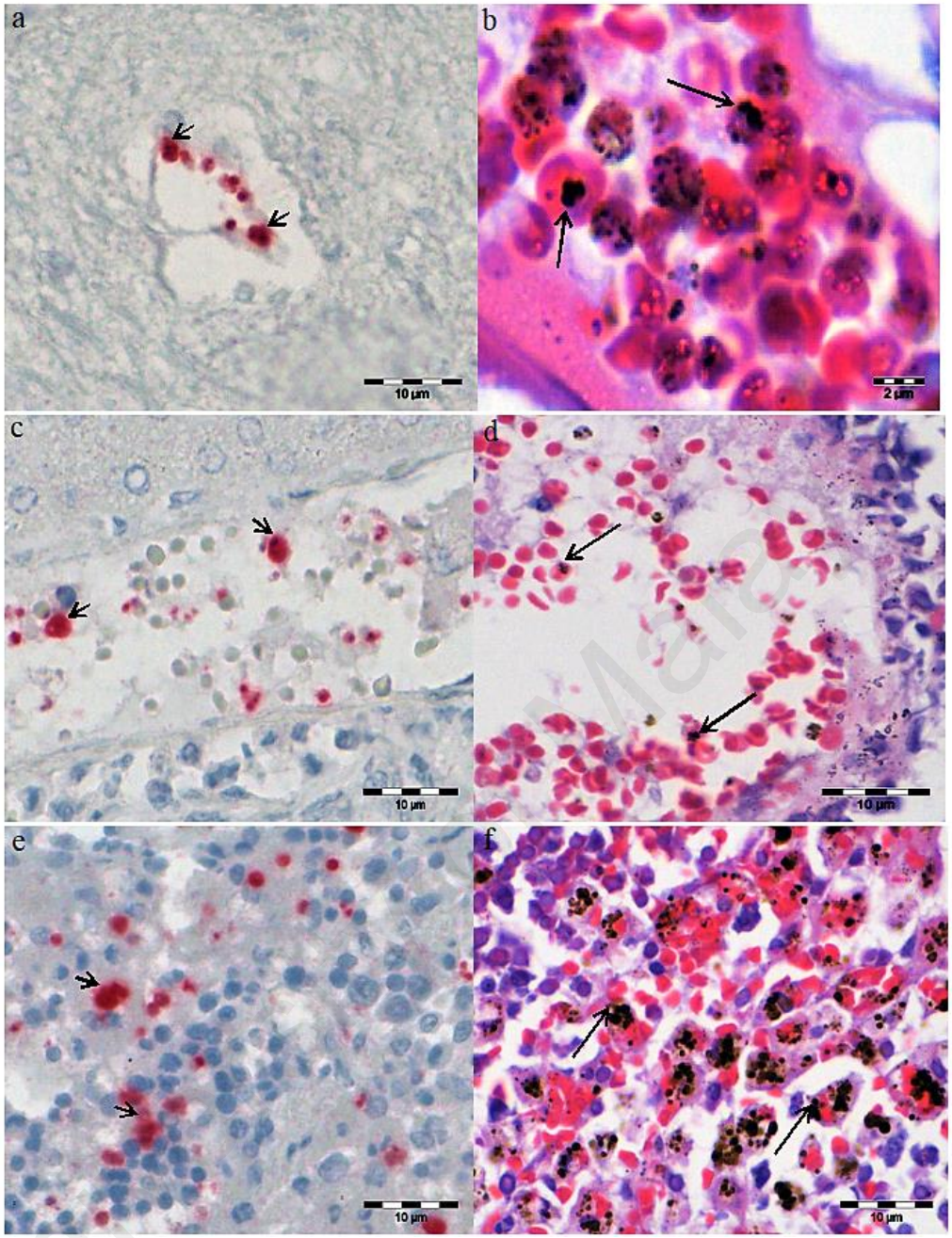

Figure 3.9: Histopathology of selected organs from infected gerbils.

a, c, and e**,** are representatives of *Plasmodium* probe *in situ* hybridization on infected brain (mg x 400), liver (mg x 600) and spleen (mg x 600) sections with depigmentation respectively. b, d, and f are representative of haematoxylin and eosin staining on infected brain (mg x 1000), liver (mg x 600), and spleen (mg x 600) sections without depigmentation, respectively. Short thick arrows show PbA-infected RBCs while long thin arrows show *Plasmodium* pigments (hemozoin). *Plasmodium* pigments (hemozoin) were removed with 10% ammonium oxide in 70% ethanol after de-paraffin on *in situ* hybridization slides. Post mortem was performed on 19 gerbils used for clinical assessment and survival tests. mg: magnification.

3.4 DISCUSSION

Malaria immunology and its underlying pathogenesis have been studied extensively, with the experimental models mostly focusing on combinations between different mouse strains and *Plasmodium* species. These studies have led to significant findings that have included identifying genes responsible for enhanced malaria survival in wide analysis of different mouse line genomes (Bopp et al., 2010), recognizing mechanisms associated with strain-specific malaria infection (Wu et al., 2014), and determining the genotypic diversity of rodent malaria parasites (Carlton et al., 2002; Hall et al., 2005; Otto et al., 2014).

However, the use of gerbils (*Meriones unguiculatus*) in parasitic infection studies has so far been limited mostly to *B. malayi* and *B. pahangi* filaria parasites (McVay et al., 1990; Porthouse et al., 2006). A previous study examined the infectivity and immunogenicity of mouse-adapted strains of *P. berghei* K173 on gerbils in the 1970s (Weiss, 1976). Previously, *P. berghei* K173 was described as causing non-cerebral malaria and death from other malaria-related complications in different mouse strains, unlike *P. berghei* ANKA which is more lethal and causes cerebral malaria (Mitchell et al., 2005; Neill & Hunt, 1992). Here, this study uses gerbil adapted to *P. berghei* ANKA (PbA) to study its effects on an immunological basis and the underlying pathology. The study showed that PbA infection causes severe malaria in gerbils in terms of loss of body weight, lowered haemoglobin concentrations and RBC counts, as well as pigmentation and enlargement of the spleen and liver.

Many factors influence the clinical outcome of malaria infection in both humans and rodents. It has been suggested that infections caused by malaria parasites can vary in virulence depending on the complexity between environmental factors and the host, as well as parasite genetics (Sanni et al., 2002; Stephens et al., 2012). The present study

showed gerbils to be highly susceptible to PbA infection, even at low dosages (10^2 and 10^3 iRBC). The susceptibility of different mouse strains to PbA infection has been characterized previously (Bopp et al., 2010). C57BL/6 and CBA mouse strains succumb to PbA-induced cerebral malaria, whereas others such as DBA and C58, are resistant to experimental cerebral malaria (ECM), dying instead because of hyper-parasitaemia and anaemia (Bopp et al., 2010; Sanni et al., 2002). The findings here showed that gerbils survived longer (11-19 days) than C57BL/6 and CBA mice (6-10 days), challenged with the same concentrations (1×10^6 iRBC) of PbA (Bopp et al., 2010; Neill & Hunt, 1992; Sanni et al., 1998). However, the survival of gerbils following PbA infection was similar to that of DBA and C58 mice, which survived for 11-18 days and 15-22 days post-infection (pi) (Bopp et al., 2010), respectively.

The high mortality rate of PbA infection in gerbils can be attributed to high parasitaemia and anaemia, which have been implicated in other non-ECM mouse models (Neill & Hunt, 1992; Sanni et al., 2002). High parasitaemia of above 40% iRBC observed in this study is in line with high parasitaemia ($\geq$ 60% iRBC) reported previously in non-ECM mouse models (Amani et al., 1998; Neill & Hunt, 1992), whereas parasitaemia $\leq$ 20% iRBC has been reported for ECM-susceptible mice (Randall et al., 2008; Sanni et al., 2002). The low parasitaemia observed in ECM mice has been described to be due to sequestration of PbA in organs such as the brain, spleen, liver and lungs, which leads to a reduced presence of the parasites in the peripheral blood (Randall et al., 2008).

Clinical symptoms such as weight loss and hypothermia were monitored. Hypothermia, defined as having a temperature below 30°C, has been associated with haemorrhage in the brain and early death in ECM-susceptible mice (Curfs et al., 1989). In addition to the suggested association between hypothermia and ECM, hypothermia below 36°C has also been identified as a marker for terminally ill rodents in an infectious

bacterial disease model (Kort et al., 1998). In a *P. chabaudi chabaudi* (AS) infection, hypothermia has been found to correlate with the parasitaemia level, where resistant or resolving (B10 knock out) mice showed no hypothermia with peak parasitaemia 20 – 30%, compared to susceptible (DBA/2 and A/J) mice, with peak parasitaemia 50 – 60% (Cross & Langhorne, 1998). The current findings in gerbils with hypothermia (below 35^{o}C) are similar to those reported by Bopp et al. (2010), in both mice susceptible and resistant to CM induced by PbA. Additionally, hypothermia in rats has been associated with increased turnover of 5-hydroxytryptamine (5-HT serotonin) in the brain, which is a putative neurotransmitter, leading to both lower food intake and lower body temperature (Dascombe & Sidara, 1994).

Overall, the data do not support the conclusion that gerbils died as a result of CM. This is due to the fact that none of the gerbils showed neurological symptoms such as ataxia, convulsion and deviation of the head. A report by Amani et al. (1998) had shown that the genetic background of the mouse affects the disease outcome of PbA infections, but also that the cloned lines of PbA differ in their ability to induce ECM. As the PbA in this study was adapted to gerbils prior to the experiments, it is possible that the ability of PbA to induce ECM was modulated.

Severe malaria anaemia (SMA) has been identified as one of the causes of mortality in ECM-resistant mouse models (Neill & Hunt, 1992). Findings here showed that gerbils suffered from severe anaemia with significant low haemoglobin (Hb) concentrations (< 3 g/dL) and total RBC counts (< 9×10^{8} RBC/mL) during the course of infection. According to the World Health Organization (WHO), the standard measurement for SMA is haemoglobin (Hb) concentration < 50 g/L or 5 g/dL (WHO, 2011). The haematocrit shown here is similar to the low haematocrit (< 10% PCV) observed in C57BL/6 mice infected with PbA and PK173 (Mitchell et al., 2005). Haemoglobin concentration and

total RBC count of <40 g/L and 20×10^8 RBC/mL, respectively have been reported during PbA infection in wild-type and knockout mice (Amani et al., 2000). The underlying mechanisms of the factors contributing to severe malaria anaemia (SMA), which include dyserythropoiesis, phagocytosis of infected and uninfected RBCs, and erythrocytic suppression, are still poorly understood (Lamikanra et al., 2007). Helegbe et al. (2009) have suggested that auto-antibodies play a potential role in the destruction of uninfected RBC in semi-immune mice. Host genetic factors may also influence the outcome of auto-immune mediated mechanisms in RBC destruction (Helegbe et al., 2009; Lamikanra et al., 2007). *Plasmodium* by-products, mainly hemozoin, have been suggested as a contributing factor for suppressed erythropoiesis, low reticulocytosis and malaria anaemia by inhibiting the proliferation of erythroid precursors (Thawani et al., 2013). It has been proposed that SMA is mediated partly by immune-pathogenic mechanisms, mostly through a hyper-activated phagocytic system which thus aids the destruction of uninfected RBCs (Evans et al., 2006). However, more molecular evidence is still required to determine the major cause of severe anaemia in *Plasmodium* infections.

The role of innate immunity as a protective response to malaria infection has been established (Riley et al., 2006; Stevenson & Riley, 2004; Wu et al., 2014). Over-production of cytokines has been implicated in the pathogenesis of severe malaria (Artavanis-Tsakonas et al., 2003; Lou et al., 2001; Schofield & Grau, 2005). Previous reports have shown that malaria infection is associated with the development of Th1 cytokine response such as IL-1, IL-6, IFN-γ and TNF-α (Hunt & Grau, 2003; Lamb et al., 2006). These studies are in agreement with the present findings, with gerbils eliciting pro-inflammatory cytokines in response to PbA. This study shows that gerbils did not respond early to PbA infection as revealed by expressions of inflammatory cytokines such as IFN-γ and TNF. Early production of IFN-γ has been suggested to correlate with protection from lethality of *P. yoelii* infection (Hunt & Grau, 2003; Lamb et al., 2006). Moreover,

late production of IFN-γ has also been suggested to be crucial in the development of CM (Grau et al., 1989). Inflammatory cytokines such as tumour necrosis factor- α (TNF-α), interleukin-1 (IL-1) and IL-6, have been described to correlate with severe malaria but the major role of TNF-α has been linked with parasite killing (Artavanis-Tsakonas et al., 2003; Kwiatkowski et al., 1990). Interferon gamma (IFN-γ) on the other hand, has been identified to be associated with pathogenesis and protection against CM (Amani et al., 2000), as well as controlling blood stage *Plasmodium chabaudi* AS (Su & Stevenson, 2000). Nonetheless, this study shows that both IFN-γ and TNF were persistently elevated for over 8 days during the 11-day time course. Hence, it can be suggested that the persistent elevation of innate immune response such as Th1 cytokines by gerbils is part of the host's immune response to eliminate the parasite.

Although elevated levels of serum IL-6 have been reported in ECM-susceptible mice (Randall et al., 2008), its role in the severity of the disease in mice is yet to be ascertained. However, in clinical studies, IL-6 has been found to be associated with hyper-parasitaemia and human CM (Day et al., 1999; Lyke et al., 2004; Wenisch et al., 1999). The present study shows IL-6 mRNA to be the least elevated in all the inflammatory cytokines measured in the plasma. This shows there is still a need to study the role of IL-6 in severe malaria.

It has been suggested that IL-10 plays an important role in immune regulation by down-regulating pro-inflammatory cytokines (such as TNF, IL-6 and IL-12), thereby inhibiting Th1 function and activities of natural killer cells (Cai et al., 1999; Lyke et al., 2004). This is further supported by a study by Li et al. (2003) on the pathology of *Plasmodium chabaudi chabaudi* in C57BL/6 mice, showing regulatory cytokines such as transformation growth factor beta (TGF-β) and IL-10 to be crucial in modulating the magnitude of immunopathology during malaria infection. More so, other studies have

suggested that the balance of anti-inflammatory to pro-inflammatory cytokines produced during *Plasmodium* infection determines the severity of malaria outcome (Baccarella et al., 2014; de Kossodo & Grau, 1993). However, more evidence is still required to determine whether the ratio or regulation of anti-inflammatory to pro-inflammatory cytokines would lead to protection or exacerbation of the host towards severe malaria.

Here, this study demonstrated that the gerbils responded to PbA infection by eliciting a combination of Th1 and Th2 cytokine responses. Immuno-regulatory cytokine such as IL-10 was significantly elevated in the three organs (blood, brain and spleen) tested during the course of infection. This might explain the reason gerbils were resistant to induced CM by PbA. The severity of anaemia has also been suggested to be dependent on levels of TNF-α relative to anti-inflammatory cytokine IL-10 (Lamikanra et al., 2007). This has also been observed in clinical studies where a low ratio of plasma TGF-β and IL-10 to TNF-α was associated with severe malaria anaemia in young children in malaria endemic communities in Africa (Achidi et al., 2013; Othoro et al., 1999).

Identification of malaria parasites in formalin fixed paraffin embedded (FFPE) tissues has always been subject to many different methods and interpretations. Results from routine haematoxylin and eosin staining require highly skilled microscopists and mostly rely on the malaria parasite's visible pigments (hemozoin) which can easily be confused with deposits or pigments from formalin or other tissue processing reagents. However, in the present study, a *Plasmodium* genus DNA probe was employed to detect the presence of PbA iRBC in different tissues. Surprisingly, the findings show PbA to be present in tissues such as the brain, liver, lungs, kidneys and spleen, and this can be visualized under lowest magnification with the aid of an *in situ* hybridization method. Previously, a chromogenic *in situ* hybridization method had proven to be a sensitive and specific tool for detection of *Plasmodium* parasites in FFPE tissues (Dinhopl et al., 2011; Dinhopl et

al., 2015; Ilgūnas et al., 2016). However, these studies were so far only conducted on avian malaria parasites, and with this study, it can be recommended that this highly powerful tool could be adopted in a human clinical setting.

Furthermore, sequestrations of malaria parasites in the tissues are considered to be critical for disease pathogenesis (Dondorp et al., 2005; Randall et al., 2008). The absence of mature trophozoites and schizonts of *P. falciparum* in human peripheral blood circulation has been suggested as evidence for sequestration of these stages (Franke-Fayard et al., 2010). Conversely, both the schizont and matured trophozoites of PbA have been found to sequester, while the ring stages and gametocytes remain in circulation (Mons et al., 1985).

Previous works have shown that accumulation of PbA-infected red blood cells (iRBCs) can be found in organs such as the brain, liver, lungs, spleen, kidneys and adipose tissues in different murine models (Amante et al., 2007; Franke-Fayard et al., 2005; Martins et al., 2009; Neill & Hunt, 1992). These reports are similar to the present findings with gerbils, where PbA iRBCs were also found to accumulate in the brain, liver, lungs, kidneys and spleen. Some researchers have suggested that the sequestration of *P. berghei* in the brain is not associated with ECM (Carvalho et al., 2000; Franke-Fayard et al., 2005), while some suggest otherwise (Amante et al., 2007; Hearn et al., 2000). Although this model shows the presence of PbA iRBCs in the brain, interestingly, no clinical symptoms of neurological effects were observed.

CHAPTER 4: *BRUGIA PAHANGI* CO-INFECTION WITH *PLASMODIUM BERGHEI* ANKA IN GERBILS PROTECT AGAINST SEVERE MALARIA

4.1 INTRODUCTION

Generally, the overall prevalence of lymphatic filariasis (LF) caused by *W. bancrofti*, *B. malayi* and *B. timori* is low, although the disease continues to pose threat to human race due to its social and economic impact, debilitating and disfiguring consequences (Brooker et al., 2007; WHO, 2016b). According to the World Health Organization (WHO), an estimated 216 million cases of malaria occurred in the year 2016 worldwide, and this includes an additional 5 million cases to the 2015 cases (WHO, 2017). WHO also estimated that 445,000 deaths occurred in the year 2016 due to malaria, and approximately 91% of these deaths occurred in the WHO African region (WHO, 2017). On the other hand, previous reports on the concomitant infection of *W. bancrofti* and malaria infection is less than 1% in India (Ghosh & Yadav, 1995; Ravindran et al., 1998), less than 4% in Guyana (Chadee et al., 2003), less than 5% in Kenya (Muturi et al., 2006), and about 18% prevalence of concomitant infection of *Mansonella perstans* and malaria was reported in Uganda (Hillier et al., 2008).

Lymphatic filariasis (LF) and malaria are common in many tropical regions, especially Africa and India subcontinent. The two are mosquito borne diseases which often share the same vectors (Manguin et al., 2010). The study by Muturi et al. (2006) has shown that concomitant infection of *W. bancrofti* and *P. falciparum* can occur naturally in both humans and female *Anopheles* mosquitoes. Similarly, multiple infections of *W. bancrofti* and *P. falciparum* have been reported in female *Anopheles* mosquitoes from Papua New Guinea (Burkot et al., 1990).

Co-infection of helminths and malaria parasites has continued to generate debate on the effects of helminth infections on the susceptibility to malaria infections and its

severity. Some studies have reported that helminth infections exacerbate the clinical outcome of malaria (Le Hesran et al., 2004; Nacher et al., 2002; Wilson et al., 2007), whereas others reported that helminth infections protect or ameliorate the severity of malaria (Briand et al., 2005; Brutus et al., 2007; Lyke et al., 2005).

Similarly, studies on co-infection of filaria nematodes and malaria parasites in animal models have also shown conflicting reports. In studies using animal model, *H. polygyrus* co-infected with *P. chabaudi* AS in C57BL/6 mice, showed that the worm parasite protects against severity of malaria pathology (Segura et al., 2009), while elsewhere, similar parasites and animal model co-infection reported that the worm infection resulted in exacerbation of malaria induced liver pathology (Helmby, 2009). Moreover, some studies have reported that there is no differences in malaria outcome among co-infected and mono infected mice (de Souza & Helmby, 2008; Tetsutani et al., 2008), whereas others have also reported that the presence of filaria infection alters the efficacy of malaria vaccine (Kolbaum et al., 2012; Su et al., 2006).

However, it has been suggested that filaria and malaria infections induce parasite-specific immune responses which is characterized by elevation of T-helper cell type 2 (Th2) and Th1 cytokines respectively. Furthermore, it has been suggested that helminth infections in human aid in the transmission of malaria parasites, whereby the host harbours more circulating gametocytes; the duration of malaria infection is increased and vectors are more attracted to the host than usual (Nacher, 2012). Study by Murray et al. (1978) showed that treatment of helminth led to an early increase in incidence of malaria, and this suggested that pre-existing or concomitant infection of filaria infection may have influence on the treatments of malaria infections and also alters the efficacy of malaria vaccines. Currently, there are series of efforts to eliminate both LF and malaria in the next decade (WHO, 2017). However, there are more concerns and questions on the impact of

antifilaria drugs on the immune responses to malaria infection in co-infection settings or endemicity, and its potential result on malaria morbidity and mortality (Muturi et al., 2008). Therefore, the main objective of this study is to establish gerbil-filaria-malaria co-infection model and evaluate the interactions that occur between the host and parasites.

4.2 MATERIALS AND METHODOLOGY

4.2.1 Maintenance of *Aedes togoi* mosquitoes

The *Aedes togoi* used in this study was the Malaysian strain that had long been established and maintained at the Department of Parasitology, University of Malaya. The eggs, larvae and pupae were cultured in 1% saline (100 g/10g of salt in 10 L/1L dechlorinated water respectively). About 250-350 larvae were maintained per tray and the rearing solution was changed every 3-4 days (depending on turbidity). The larvae were fed with compounded food which comprises of beef liver, mice feed and yeast. The pupae were isolated in the cages as they emerged and the adults were fed 10% glucose solution.

4.2.2 Parasites and infections

The filariae, *B. pahangi* were obtained from an infected cat in Carey Island, Klang, Selangor, Malaysia. The filaria infected blood was fed to *Ae. togoi* mosquitoes through membrane feeding. Fully or partially fed mosquitoes were separated into cups and incubated at 27ºC and 89% humidity in a humidity chamber, for 12 post infection days. The mosquitoes were then dissected by crushing in cold phosphate buffered saline (PBS), after which the active infective larvae (L3) were sorted under a dissecting microscope. About 100 L3 in 200 µL phosphate buffered saline (PBS) were inoculated subcutaneously in donor gerbils (Figure 4.1). After 70 days patency period, about 40-60 µL of blood were collected through tail prick from the gerbil and a thick blood film was then prepared. The

blood film was allowed to dry for 24 hrs, after which Giemsa stain was applied and viewed under light microscope for confirmation of infection.

The protocols and methods for the *Plasmodium* infections have been elaborated in Chapter 3. Briefly, a PbA pRBCs (200 µL) from stocks were inoculated intraperitoneally into naïve gerbil. After 5-7 days post infection or parasitemia above 15%, the donor gerbil was then sacrificed and the harvested pRBCs was passaged in experimental gerbils at dosage of 10^6 pRBCs in 200 µL of PBS.

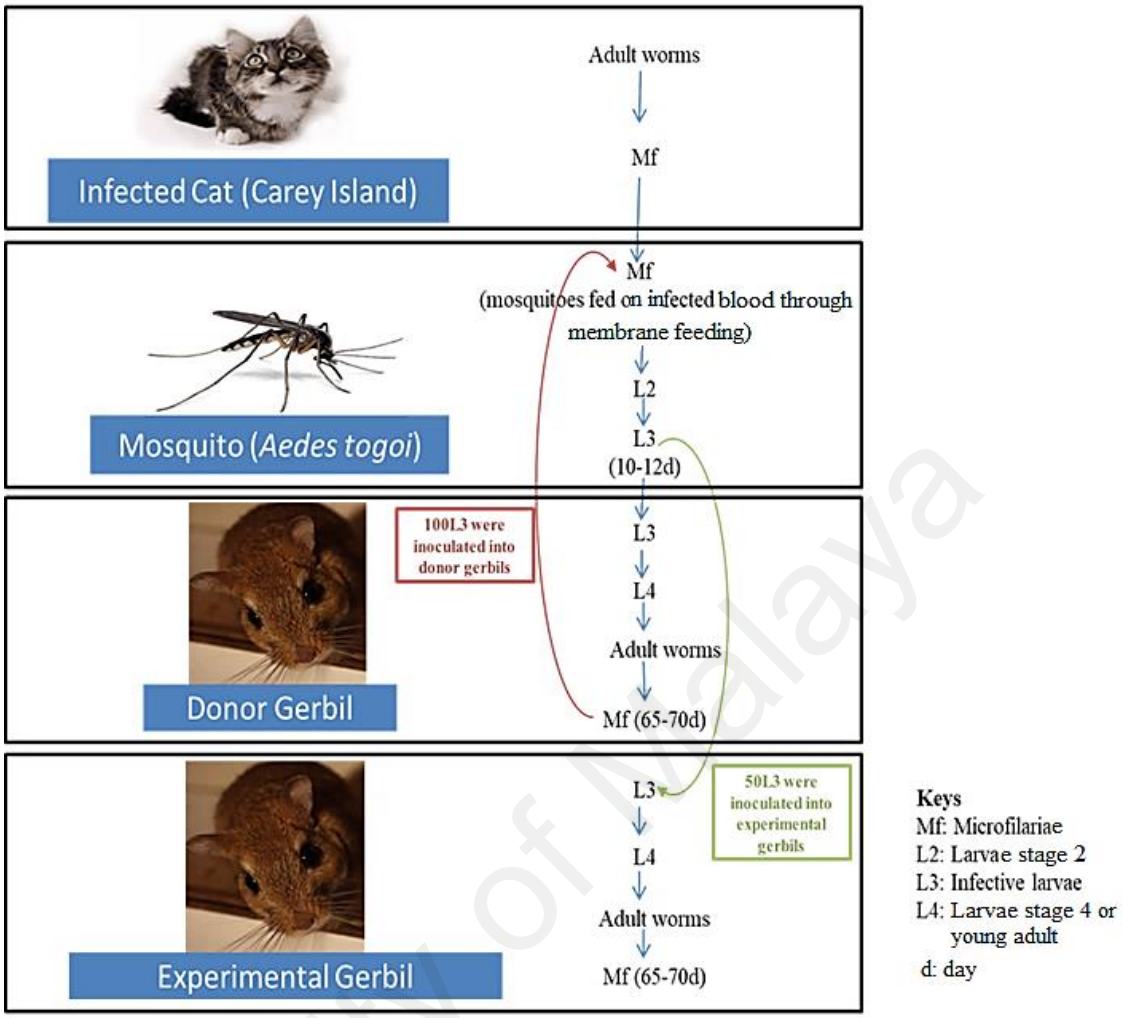

Figure 4.1: Maintenance and infection of *Brugia pahangi* in both mosquito and gerbil.

4.2.3 Experimental infections

Gerbils were divided into four groups (Figure 4.2). Group 1 comprised of uninfected gerbils in which PBS was administered subcutaneously at day 0 and intraperitoneally at day 70 post inoculation. Group 2 gerbils were inoculated with 50 L3 of *B. pahangi* in 200 µL PBS subcutaneously into the left lumbar area of each gerbil, and after 70 days post infection (pi), 200 µL PBS was administered intraperitoneally. Group 3 gerbils were given 200 µL PBS subcutaneously at day 0, and after 70 days (filaria patency period), 10^6 PbA infected red blood cells (iRBCs) were administered intraperitoneally. Group 4 gerbils were inoculated with 50 L3 *B. pahangi* in 200 µL PBS subcutaneously into the left lumbar area at day 0, and after 70 days pi, 10^6 PbA infected red blood cells (iRBCs) were administered intraperitoneally.

4.2.4 Disease assessment

The animals were monitored daily to quantify their water and food intake. To evaluate their survival rate, gerbils were monitored daily for a period of 30 days. Gerbil's body weight was determined by using electronic weighing balance (A&D, Japan) and their body temperature was measured orally with thermometer (Rossmax TG380, Switzerland). The haemoglobin concentration of gerbil was measured with Hemocue AB (Angelholm, Sweden) by pipetting 5-10 µL of tail blood into the cuvette, and readings were taken. The blood glucose concentration was determined by Accu-CHEK performa (Roche, Mannheim Germany). About 5 µL of blood from tail prick was dropped on the probe of the strips and reading was taken after few seconds.

Keys

PBS:	Phosphate Buffer Saline	Temp: Temperature
L3:	Infective larvae	Glu: Glucose
iRBCs:	Infected red blood Cells	N: Number of gerbils
BW:	Body Weight	
Hb:	Haemoglobin	

Figure 4.2: Experimental infections

4.2.5 Immune responses of gerbil

4.2.5.1 RNA extraction and cDNA preparation

The animals were euthanized with over dose of ketamine (Troy Laboratories, Australia) and xylazine (Santa Cruz Animal Health, USA) intraperitoneally at the dose of 150 mg/kg and 6 mg/kg respectively (Hrapkiewicz et al., 2013). About 50-100 mg of the spleen and brain were surgically removed and the tissues snapped frozen in liquid nitrogen before extracting the total RNA with TRIzol reagent (Life technologies, USA). Briefly, 1 mL TRIzol reagent was added to the tissue, then homogenized with hand-held rotor 2-3 mins and was incubated for 5 mins at room temperature. Chloroform, 0.2 mL was added and shaken vigorously for 15 secs and incubated for 2-3 mins at room temperature. The samples were then centrifuged at 12,000 × g for 15 mins at 4^{o}C. About 400 µL of the aqueous layer was removed and 0.5 mL of cold isopropanol was added and mix. The RNA was allowed to precipitate for at least 2 hrs at 4^{o}C and then span at 12,000 × g for 30 mins at 4^{o}C. The samples were washed with 75% ethanol and allowed to dry for 3-5 mins at room temperature. About 70 µL of DEPC water was then used to elute the RNA and heat the sample at 60^{o}C for 10 mins. The RNAs were stored at -80^{o}C until further use.

Total RNA of 2 µg was then converted to cDNA with Super Script IV first-strand synthesis kit (Thermo Fisher Scientific, USA). The cDNA was then used as template for the RT-PCR.

4.2.5.2 Total RNA sample quality check

The RNA degradation and contamination were monitored on 1% agarose gels, while RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). Concentration of the RNA was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA). The RNA integrity number (RIN) was

assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA) (Appendix C).

4.2.5.3 Primers and probes

Gene-specific oligonucleotide primers and probes for gerbil IL-4 (Gen Bank L37779), IL-6, IL-10 (Gen Bank L37781), IFN-γ (Gen Bank L37782), TNF (Gen Bank AF171082.1) and GAPDH (Gen Bank AB040445.1), have been published previously (Yamaoka et al., 2005) and the full details of the sequence has been stated in chapter three (Table 3.1).

4.2.5.4 Analysis of cytokines by real-time PCR

Taqman PCR reactions for cytokines mRNA and the housekeeping GAPDH mRNA levels were performed using an Applied Biosystems StepOnePlus Real-Time PCR system (Life technologies, USA). A Taqman fast advanced master mix (Life technologies, USA) was used to prepare a 20 µL Taqman reactions. Briefly, 2 µL of cDNA, 10 µL of Taqman master mix, 7 µL of RNase and DNase free water, and 1 µL of probe were mixed in the 20 µL qPCR reaction. The reactions were prepared in triplicates for each sample. Comparative standard curves were generated as a result, with the data being presented as the mean fold change of the cytokines mRNA relative to the level of GAPDH mRNA.

4.2.6 Statistical analysis

All data were analysed using GraphPad prism 6. The distribution of the data was assessed by a Kolmogorov-Smirnov test for normality testing. Multiple t-test with Sidak-Bonferroni was used to analyse data for the clinical assessment, while two-way ANOVA was used to analyse data generated from the cytokines profiling experiment. Log rank test was used to analyse survival trend and rates. All results are expressed as mean $\pm$ S.E.M (Standard Error of Mean) and considered statistically significant when $p < 0.05$.

4.3 RESULTS

4.3.1 Survival of gerbils to co-infection of *Plasmodium berghei* ANKA and *B. pahangi*

Gerbils were grouped into control, *Brugia pahangi* (Bp) infection, *Plasmodium berghei* ANKA (PbA) infection, and PbA and Bp co-infection. About 50 infective larvae (L3) were inoculated subcutaneously into Bp-infected gerbils, 10^6 iRBCs were infected intraperitoneally into PbA-infected gerbils, while co-infected gerbils were initially inoculated subcutaneously with 50 L3, then after 70 days of prepatent period co-infected with 10^6 pRBCs intraperitoneally.

Expectedly, 100% of the control gerbils and Bp infected gerbils survived over 30-day experimental period. The median survival for PbA infected gerbil was 9 days, whereas it took a median of 19 days for co-infected to die (Figure 4.3a). However, the survival curve comparison showed that co-infected gerbils significantly ($\chi_{(3)}$ = 13.440, P = 0.0002) survived longer than PbA infected gerbils (Figure 4.3a). Though, all gerbils subjected to PbA infections succumbed to malaria infection and died within experimental period, except for one gerbil in the co-infected group which survived over 30 days and later died.

Moreover, PbA parasitaemia was significantly higher ($t_{(116)}$ = 5.283, P < 0.0001) in PbA infected gerbils than co-infected gerbils (Figure 4.3b). The maximum parasitaemia recorded among co-infected gerbils was less than 30%, while over 50% parasitaemia was observed in PbA infected gerbils.

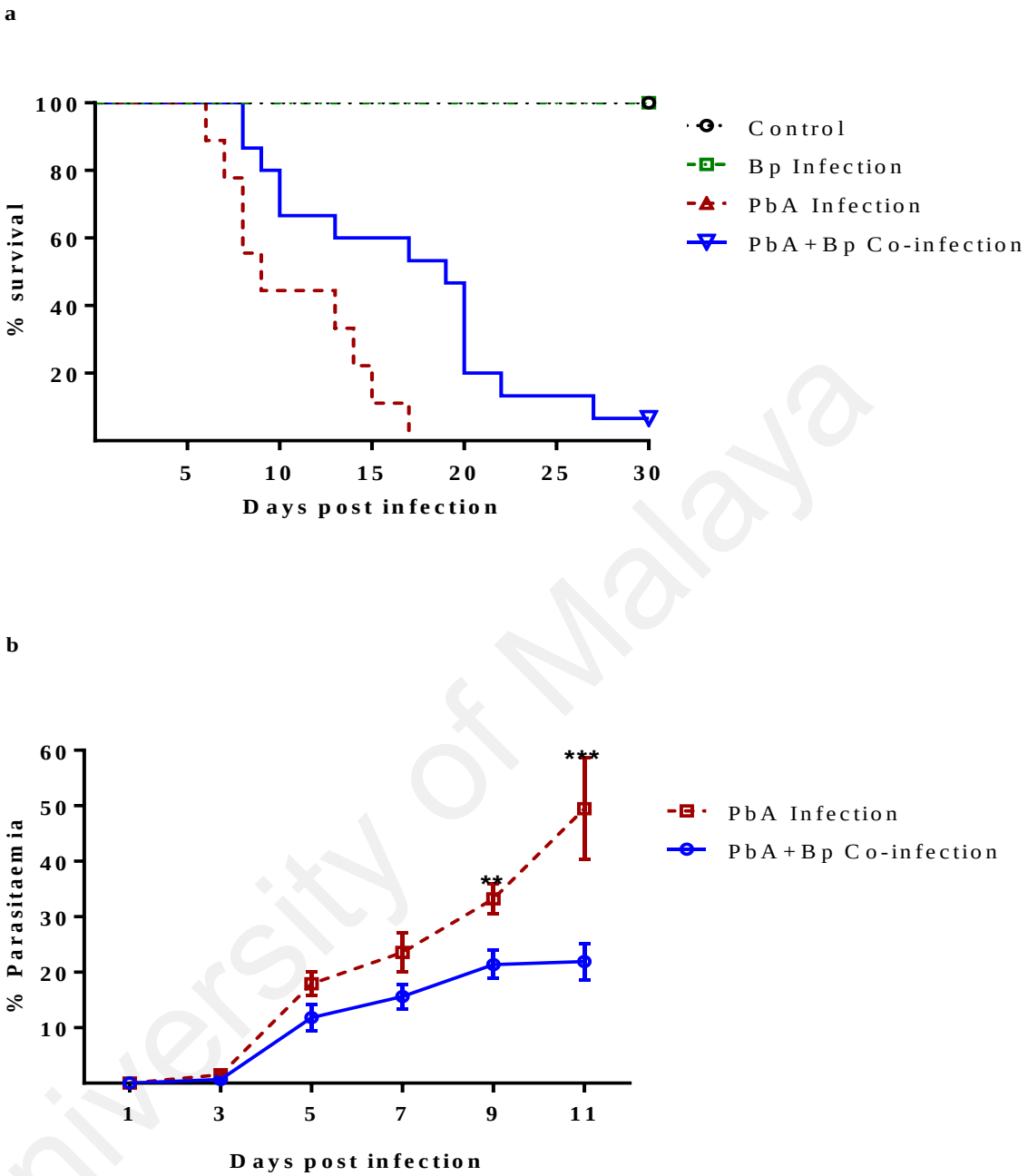

Figure 4.3: Parasitaemia and survival of gerbils to mono and co-infection.

Gerbils were infected with both mono infection (*B. pahangi* and PbA) and co-infection of *B. pahangi* and PbA. Survival and parasitaemia were monitored daily and every 2 days (respectively). **a.** Survival of gerbils to both mono and co-infection over 30 days post PbA infection. **b.** Parasitaemia level of PbA in both mono and co-infected gerbils, given 10^6 iRBC of PbA, over 11 days post PbA infection time course. Lines represent mean ± S.E.M., N= 9 per uninfected control and filaria group, while N= 15 per malaria and co-infected group. Logrank (Mantel-cox) test was used to compare survival curves. Multiple t test was used to compare the differences in the level of parasitaemia between the groups (** $P < 0.005$ and *** $P < 0.0001$).

4.3.2 Loss of appetite by PbA-infected and co-infected gerbils

During the course of the experiment, quantity of food and water consumed by gerbils were monitored daily. Table 4.1 shows that the quantity of food consumed by both PbA and co-infected gerbils reduced over time, while the food consumed by both uninfected and Bp infected gerbils remained steady. The loss of appetite for food became apparent from day 7 post infection (pi), where PbA infected gerbils consumed less than 1 g of food ($t_{(8)}$ = 13.8847, $P < 0.0001$) and co-infected gerbils consumed about 1.45 g of food ($t_{(8)}$ = 6.8908, $P = 0.0001$), compared to 5.58 g of food consumed by uninfected gerbils or 5.12 g of food consumed by Bp infected gerbils (Table 4.1). By day 11 pi, PbA infected gerbils only consumed about 0.15 g of food ($t_{(8)}$ = 11.9349, $P < 0.0001$) while co-infected gerbils consumed about 0.25 g of food ($t_{(8)}$ = 11.2839, $P < 0.0001$), compared to average 5.79 g of food consumed by uninfected gerbils (Table 4.1).

In addition, water consumption by co-infected gerbils (12.74 mL) was significantly higher ($t_{(8)}$ = 2.7752, $P = 0.0241$) at day 1 pi, compared to about 10 mL of water consumed by uninfected gerbils (Table 4.2). However, by day 9 pi, water consumption level of both PbA infected gerbils (5.40 mL) and co-infected gerbils (5.57 mL) were significantly reduced ($t_{(8)}$ = 7.9546, $P < 0.0001$; $t_{(8)}$ = 4.6541, $P = 0.0016$ respectively) compared to about 10.17 mL of water consumed by uninfected gerbils (Table 4.2).

Table 4.1: Quantification of food consumed by gerbils during infections

Days	Control (g)	Bp infection (g)	PbA infection (g)	Bp + PbA co-infection (g)	*P*-value control vs PbA	*P*-value control vs Bp+PbA
D0	5.35 ± 0.40	4.96 ± 0.37	5.31 ± 0.25	4.92 ± 0.62	0.9443	0.5765
D1	5.6 ± 0.47	4.61 ± 0.32	5.06 ± 0.54	4.80 ± 0.49	0.4716	0.2768
D3	5.82 ± 0.35	5.12 ± 0.35	5.79 ± 0.36	4.86 ± 0.48	0.9647	0.1447
D5	5.06 ± 0.09	4.89 ± 0.08	2.87 ± 0.83	4.49 ± 0.55	0.0307*	0.3296
D7	5.58 ± 0.18	5.12 ± 0.33	0.72 ± 0.30	1.45 ± 0.57	< 0.0001*	0.0001*
D9	5.03 ± 0.31	4.77 ± 0.48	0.17 ± 0.13	0.39 ± 0.17	< 0.0001*	< 0.0001*
D11	5.79 ± 0.45	4.59 ± 0.32	0.15 ± 0.13	0.25 ± 0.19	< 0.0001*	< 0.0001*

* Significant, Bp: *Brugia pahangi*, PbA: *Plasmodium berghei* ANKA, g: grams, N=5

Table 4.2: Quantification of water consumed by gerbils during infections

Days	Control (mL)	Bp infection (mL)	PbA infection (mL)	Bp + PbA co-infection (mL)	*P*-value control vs PbA	*P*-value control vs Bp+PbA
D0	10.44 ± 0.55	10.27 ± 0.22	10.32 ± 0.84	10.24 ± 0.74	0.9117	0.8322
D1	9.95 ± 0.52	10.53 ± 0.48	10.25 ± 0.43	12.74 ± 0.86	0.6600	0.0241*
D3	10.20 ± 0.25	10.71 ± 1.19	9.69 ± 0.34	12.99 ± 1.16	0.2604	0.0461
D5	10.67 ± 1.00	10.25 ± 0.65	9.09 ± 0.93	12.83 ± 1.14	0.2777	0.1938
D7	10.34 ± 0.71	10.29 ± 0.34	8.27 ± 0.53	6.38 ± 1.09	0.0481	0.0158*
D9	10.17 ± 0.50	10.85 ± 0.09	5.40 ± 0.34	5.57 ± 0.85	< 0.0001*	0.0016*
D11	10.74 ± 1.16	9.75 ± 0.35	4.97 ± 0.88	5.72 ± 1.42	0.0042*	0.0253*

* Significant, Bp: *Brugia pahangi*, PbA: *Plasmodium berghei* ANKA, N=5

4.3.3 Effects of prepatent filaria infections on gerbils infected with *Plasmodium berghei* ANKA

A comparison in all the groups of gerbils was conducted to monitor the clinical manifestations of both mono and co-infections in the gerbil host. Throughout the experimental period, the temperature of control and Bp infected gerbils was in the range of 38.5 and 36.5 ^{o}C, whereas the temperature observed in both PbA infected and co-infected gerbils was significantly lowered ($t_{(21)}$ = 2.297, P = 0.032) (Figure 4.4a). Moreover, there was a significant increase ($t_{(21)}$ = 4.928, P < 0.0001) in body weight of PbA infected gerbils at early stage of infection compared to co-infected-gerbils, losing weight steadily over the observational period (Figure 4.4b). The body weight of Bp infected and control gerbils remained steady and slightly increased over same period.

The effect of the infections on haemoglobin (Hb) concentrations in the blood of gerbils was also monitored. The Hb concentration observed in the control group was between 13 -15 g/dL, while that of Bp infected gerbils was between 11 and 13 g/dL (Figure 4.5a). However, there was a significantly lowered ($t_{(14)}$ = 3.612, P == 0.007) Hb concentration (as low as 2 g/dL) in PbA infected gerbils compared to co-infected gerbils with lowest Hb concentration of 5 g/dL at day 11 post infection (Figure 4.5a). In addition, the glucose concentration in the blood of gerbils showed that the control, Bp infected and co-infected gerbils all have glucose concentrations in the range of 5.0 -7.0 g/dL, whereas the glucose concentration in the blood of PbA infected gerbils is significantly lowered ($t_{(14)}$ = 5.029, P < 0.0001) to about 1.5 g/dL (Figure 4.5b).

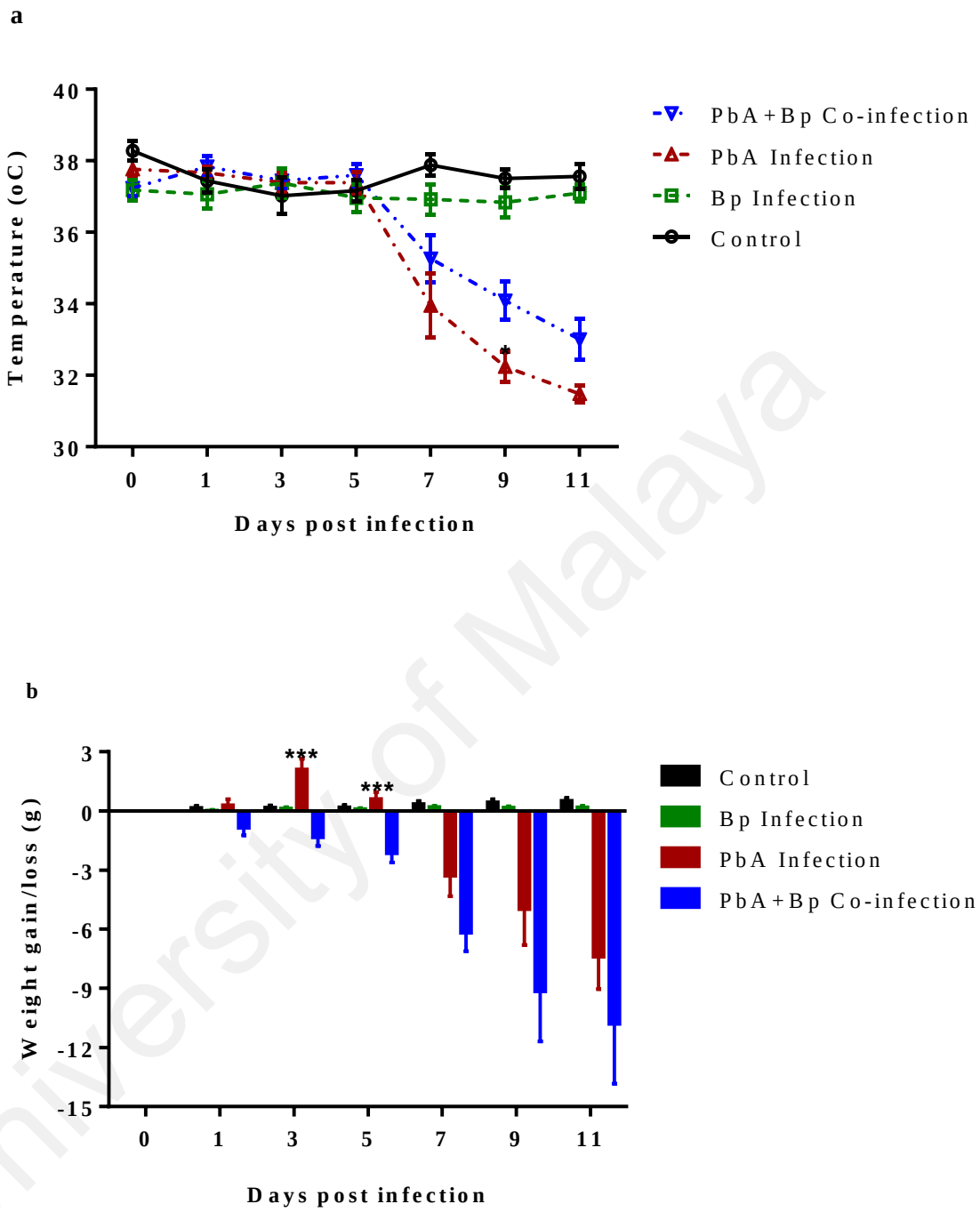

Figure 4.4: Changes in body temperature and body weight of gerbils during mono and co-infection.

a. Body temperature as measured in PbA, filaria, co-infected and uninfected control group. b. Body weight gain/loss after PbA infection. Lines and bars represent mean ± S.E.M, while N= 9. All data are representative of three independent experiments and compared by multiple t- test with Sidak-Bonferroni method used for differences between groups (*P < 0.05; ***P < 0.0001).

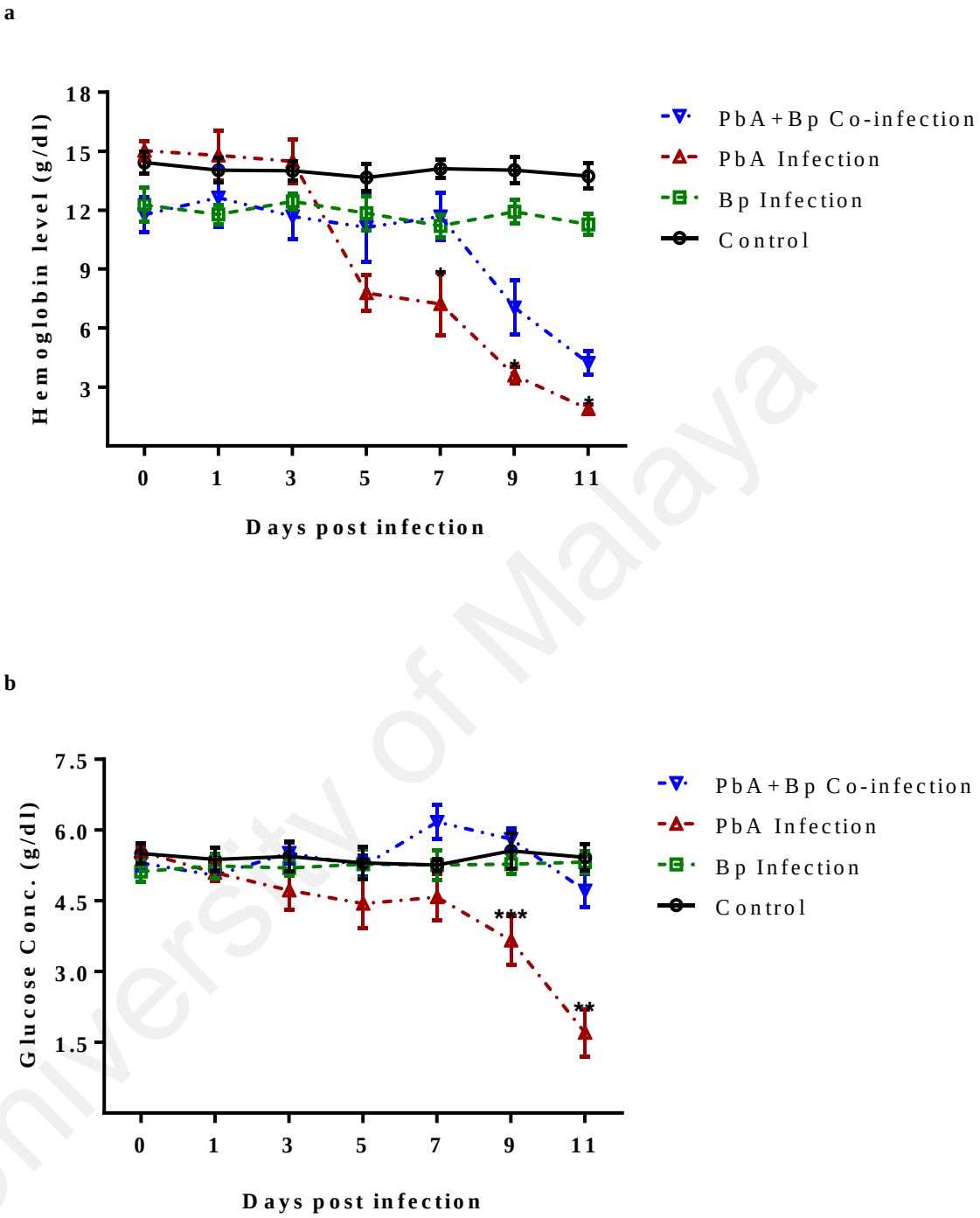

Figure 4.5: Haemoglobin and blood glucose concentration in mono and co-infected gerbils.

a. Haemoglobin level as measured in PbA, filaria, co-infected and control group b. Blood glucose as measured in PbA, filaria, co-infected and control group. Lines represent mean ± S.E.M, while N= 9. All data are representative of three independent experiments and compared by multiple t- test with Sidak-Bonferroni method used for differences between groups (*P < 0.05; **P < 0.005; ***P < 0.0001).

4.3.4 Immune responses of gerbils to co-infection of *Plasmodium berghei* ANKA and *Brugia pahangi*

The immune responses of gerbils to both mono and co-infections were monitored over a time course of 11 days. The mRNA level in both the spleen and brain were evaluated for IL-4, IL-10, IL-6, IFN-γ, and TNF, using Taqman-StepOnePlus qPCR.

The IL-4 level in the spleen of co-infected gerbils at day 7 post infection (pi) was highly significant ($F_{(6, 48)} = 8.545$, $P < 0.0001$) than any other time points, with a mean fold change of about 70 (Figure 4.6a). The IL-4 mRNA as expressed by PbA infected gerbils was mostly at low levels but was slightly higher than the expression from co-infected gerbils at day 5 pi (Figure 4.6a). However, the expression of IL-4 mRNA in the brain of both PbA and co-infected gerbils were almost the same over the time course, except for higher expression at day 7 pi by co-infected gerbils than infected gerbils (Figure 4.6b).

There was elevation of IL-10 mRNA in the spleen of both PbA infected and co-infected gerbils at different time points. The highest elevation of IL-10 mRNA in the spleen of co-infected gerbils was about 14 folds, followed by 10 folds at days 7 and 9 pi respectively, whereas the highest elevation of IL-10 mRNA in the spleen of PbA infected gerbils was 8 folds at day 7 pi (Figure 4.7a). Moreover, the expression of IL-10 mRNA level in the brain of the infected gerbils showed higher elevation of 24 and 24 folds at days 5 and 7 pi respectively, by co-infected gerbils (Figure 4.7b).

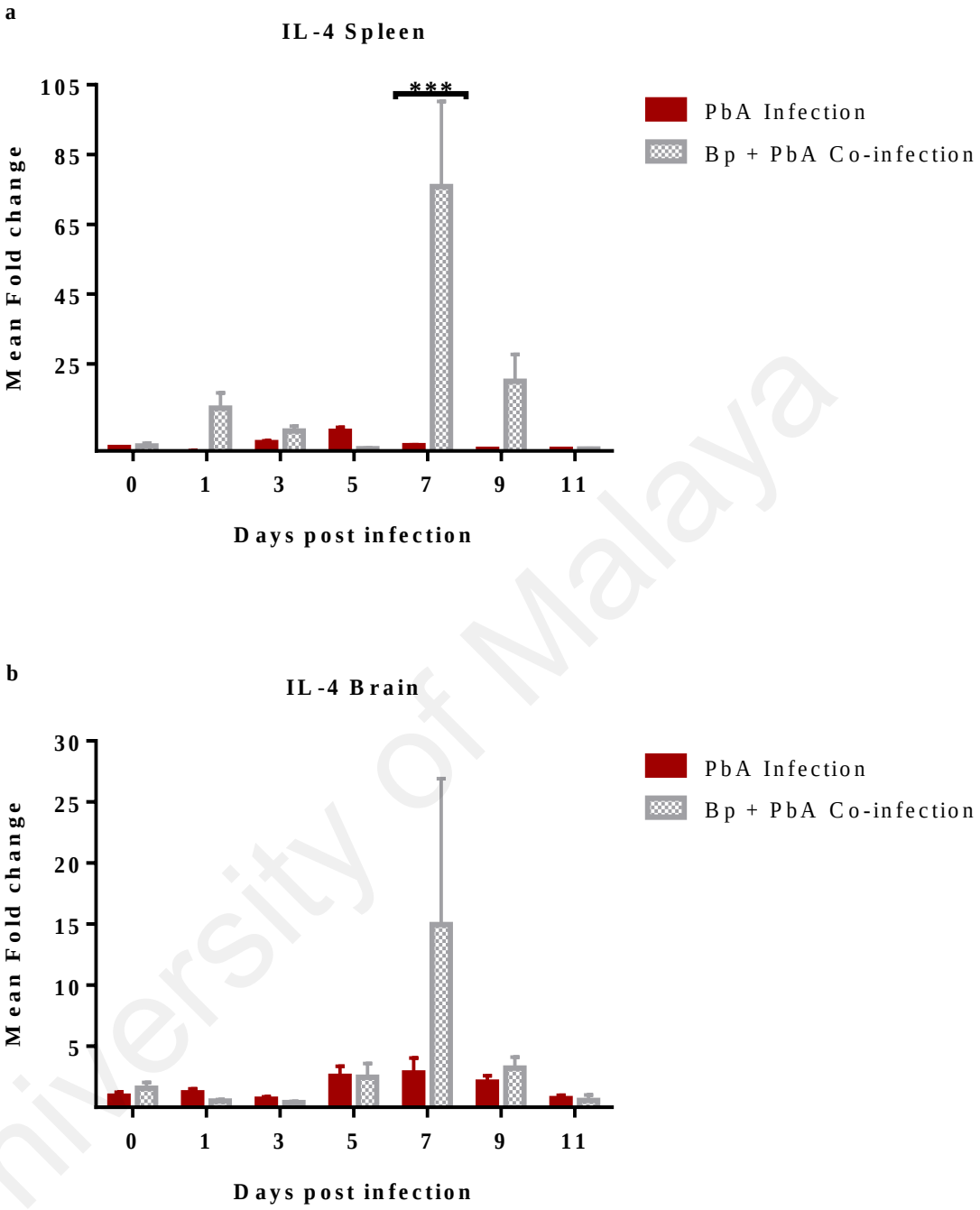

Figure 4.6: Quantitation of IL-4 mRNA in spleen (a) and brain (b).

Gerbils were euthanized under anesthesia at days 0, 1, 3, 5, 7, 9 and 11 after intraperitoneal inoculation with PbA. mRNA levels were measured by reverse transcription (RT)-PCR, and values were expressed as mean ± S.E.M (N= 5) fold changes compared with values from uninfected gerbils in the case of PbA infection, and filaria in case of co-infected gerbils. All data are representative of two independent experiments and compared by two-way analysis of variance (ANOVA) with Sidak's multiple comparison test for differences between groups.

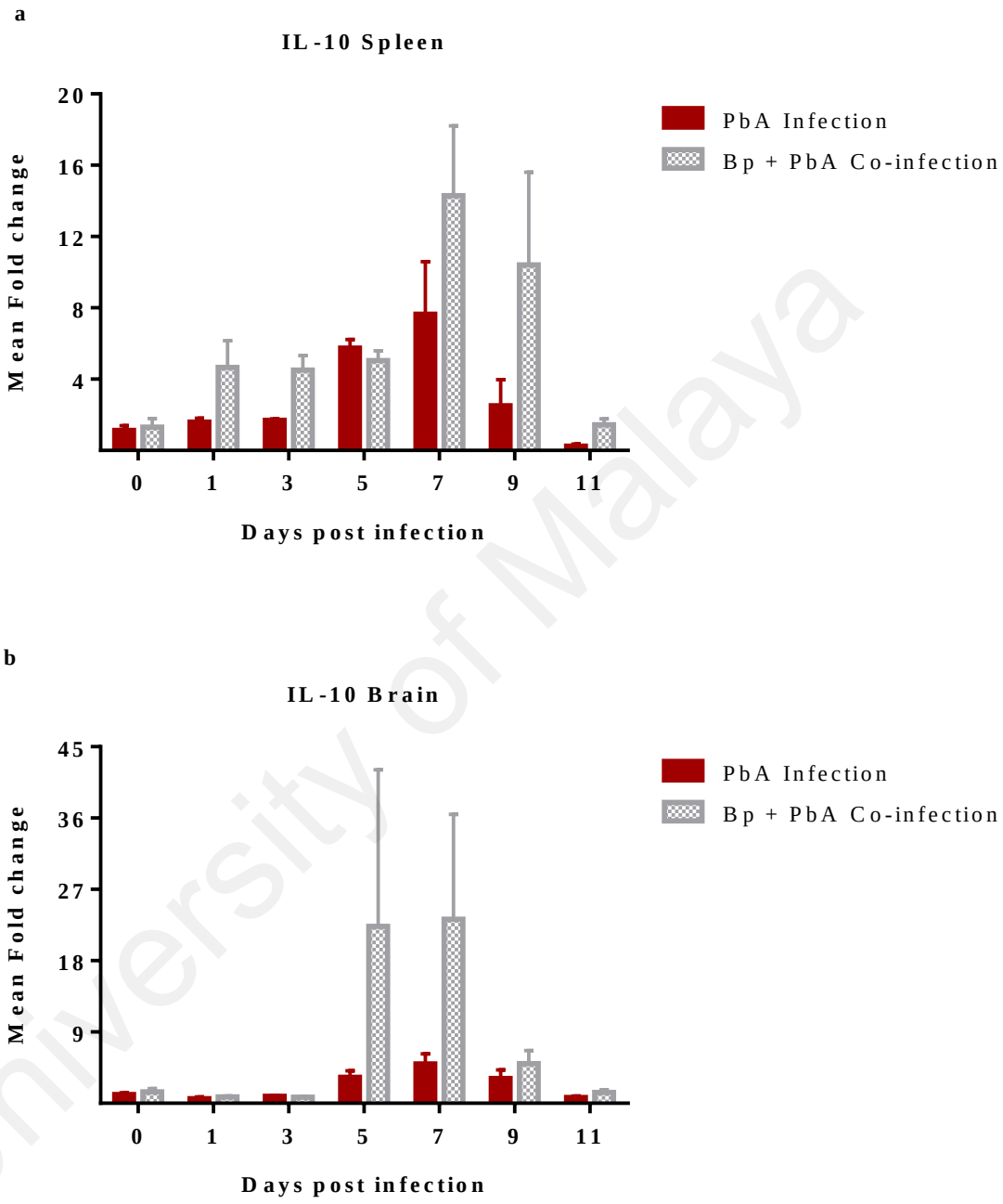

Figure 4.7: Quantitation of IL-10 mRNA in spleen (a) and brain (b).

Gerbils were euthanized under anesthesia at days 0, 1, 3, 5, 7, 9 and 11 after intraperitoneal inoculation with PbA. mRNA levels were measured by reverse transcription (RT)-PCR, and values were expressed as mean (± S.E.M, N= 5) fold changes compared with values from uninfected gerbils in the case of PbA infection, and filaria in the case of co-infected gerbils. All data are representative of two independent experiments and compared by two-way analysis of variance (ANOVA) with Sidak's multiple comparison test for differences between groups.

IL-6 mRNA in the spleen of co-infected gerbils was elicited throughout the course of experiment. At days 7 and 9 pi, about 69 and 75 folds (respectively) of IL-6 mRNA was significantly ($F_{(6, 48)} = 5.724$, $P < 0.001$) elevated than any other time points in co-infected gerbils (Figure 4.8a). IL-6 mRNA in PbA infected gerbils was slightly elevated by 6 and 15 folds at days 3 and 5 pi respectively (Figure 4.8a). Although, IL-6 was highly expressed in the brain of co-infected gerbils at day 5 (13 folds), day 7 (20 folds) and day 9 (7 folds), but these increase were not significantly different ($F_{(6, 48)} = 0.707$, $P = 0.646$) to 4, 3 and 1 folds expressed over the same time point by PbA infected gerbils (Figure 4.8b).

The IFN-γ mRNA was most elicited in the spleen of co-infected gerbils with 55 folds, followed by 52 folds and 50 folds at days 9, 7 and 3 pi respectively, and these elevations were significant ($F_{(6, 48)} = 2.943$, $P < 0.05$) than the expressions made by PbA infected gerbils over the same time (Figure 4.9a). Moreover, IFN-γ expressed with about 29 folds in the brain of co-infected gerbils was significantly higher ($F_{(6, 48)} = 1.405$, $P = 0.033$) than the 7 folds expressed in the brain of PbA infected gerbils (Figure 4.9b).

Conversely, PbA infected gerbils showed the most elevated TNF mRNA in the spleen as 11 folds, which is significantly ($F_{(6, 48)} = 11.120$, $P = 0.001$) expressed than the 4 folds observed in the spleen of co-infected gerbils at the same time, day 7 pi (Figure 4.10a). However, there were early elevations of TNF at days 1 and 3 pi (10 folds each) which were highly significant ($F_{(6, 48)} = 11.12$, $P < 0.0001$) than expressions from the spleen of co-infected gerbils (Figure 10a). Similarly, PbA infected gerbils showed that most elevated (36 folds) TNF in the brain at day 7 pi, while co-infected gerbils significantly ($F_{(6, 48)} = 3.714$, $P = 0.0493$) expressed TNF in their brain at day 5 pi with 26 folds (Figure 4.10b).

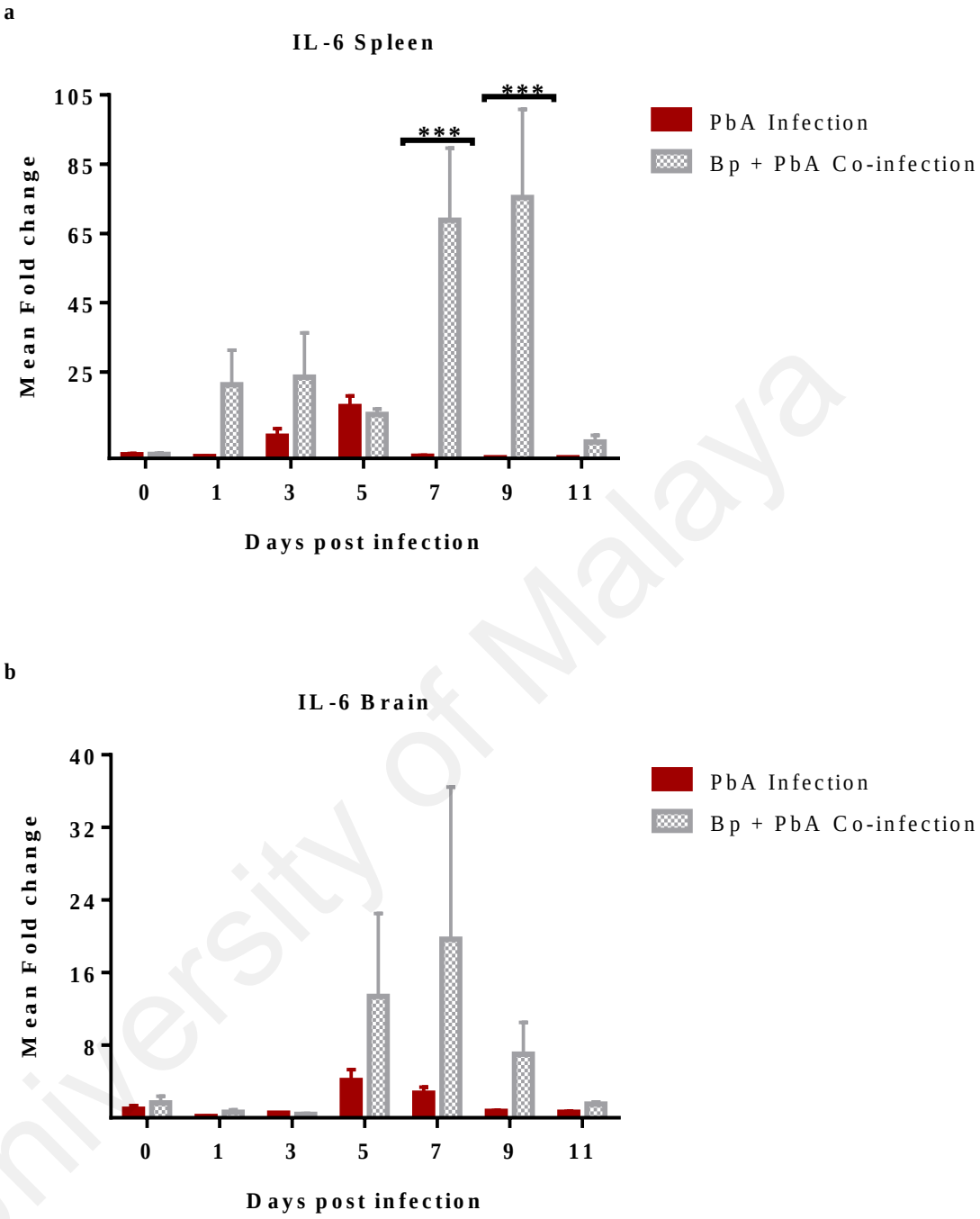

Figure 4.8: Quantitation of IL-6 mRNA in spleen (a) and brain (b).

Gerbils were euthanized under anesthesia at days 0, 1, 3, 5, 7, 9 and 11 after intraperitoneal inoculation with PbA. mRNA levels were measured by reverse transcription (RT)-PCR, and values were expressed as mean (± S.E.M, N= 5) fold changes compared with values from uninfected gerbils in the case of PbA infection, and filaria in the case of co-infected gerbils. All data are representative of two independent experiments and compared by two-way analysis of variance (ANOVA) with Sidak's multiple comparison test for differences between groups.

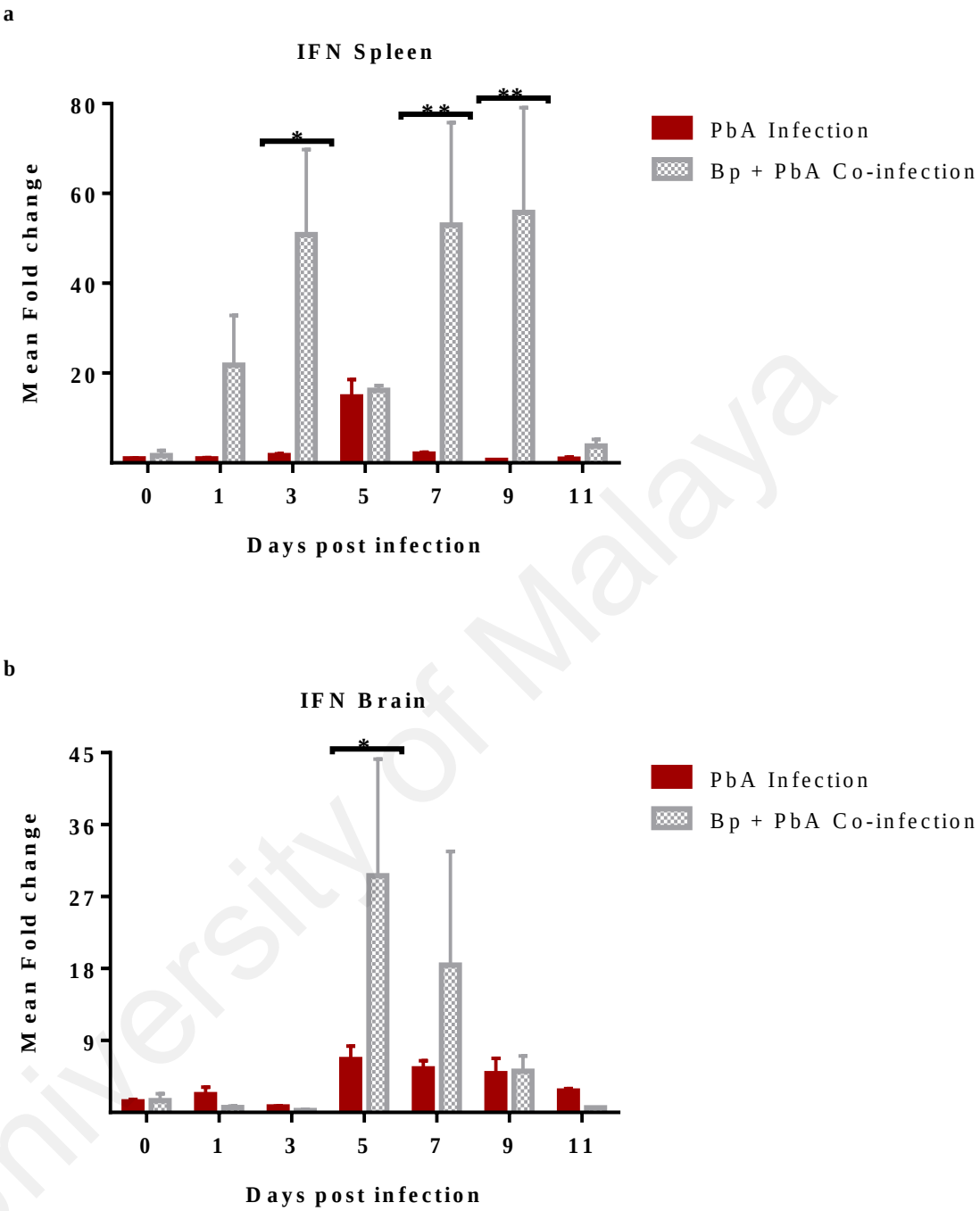

Figure 4.9: Quantitation of IFN mRNA in spleen (a) and brain (b).

Gerbils were euthanized under anesthesia at days 0, 1, 3, 5, 7, 9 and 11 after intraperitoneal inoculation with PbA. mRNA levels were measured by reverse transcription (RT)-PCR, and values were expressed as mean (± S.E.M, N= 5) fold changes compared with values from uninfected gerbils in the case of PbA infection, and filaria in the case of co-infected gerbils. All data are representative of two independent experiments and compared by two-way analysis of variance (ANOVA) with Sidak's multiple comparison test for differences between groups.

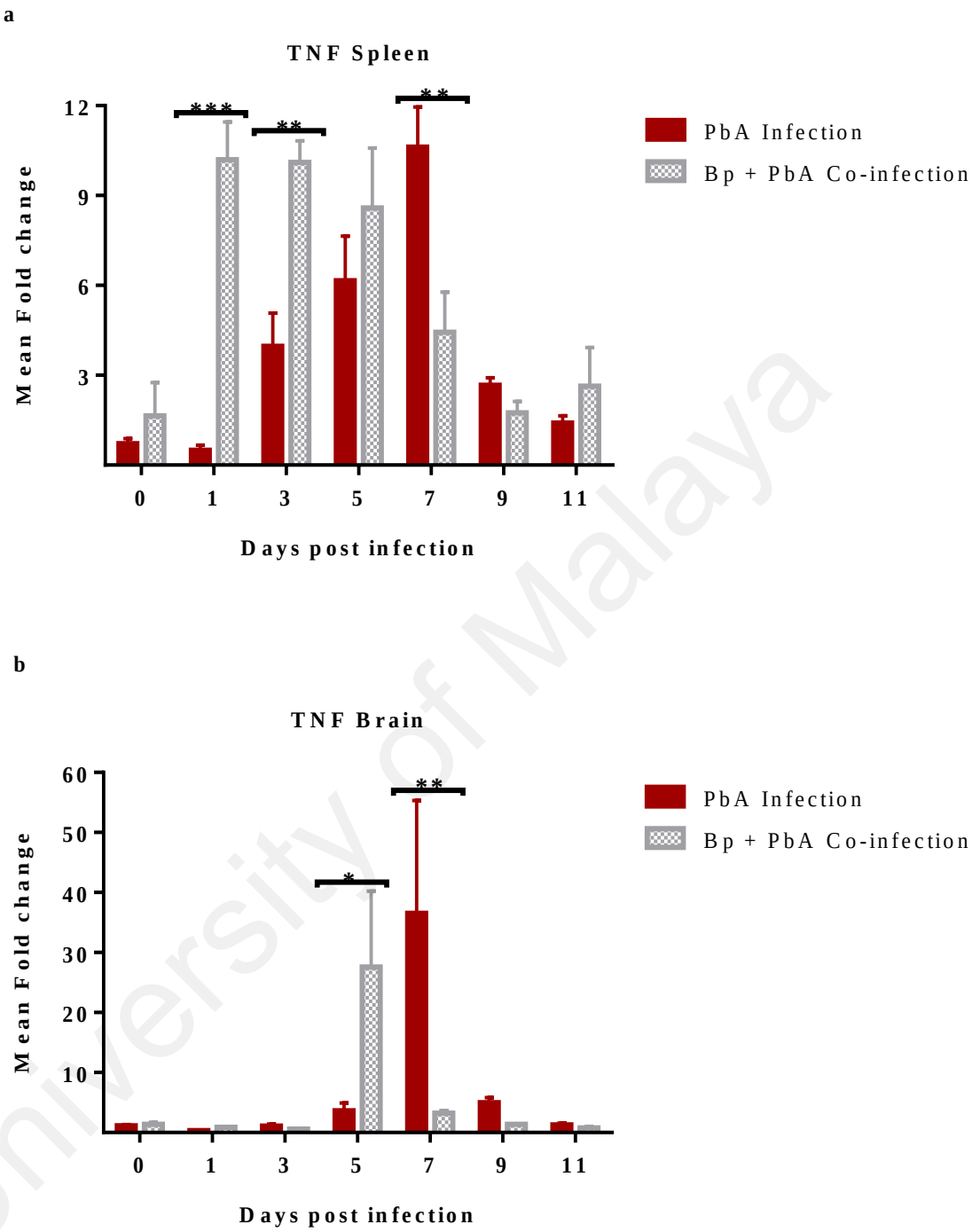

Figure 4.10: Quantitation of TNF mRNA in spleen (a) and brain (b).

Gerbils were euthanized under anesthesia at days 0, 1, 3, 5, 7, 9 and 11 after intraperitoneal inoculation with PbA. mRNA levels were measured by reverse transcription (RT)-PCR, and values were expressed as mean (± S.E.M, N= 5) fold changes compared with values from uninfected gerbils in the case of PbA infection, and filaria in the case of co-infected gerbils. All data are representative of two independent experiments and compared by two-way analysis of variance (ANOVA) with Sidak's multiple comparison test for differences between groups.

4.4 DISCUSSION

Previously, murine models on co-infection of filaria and malaria parasites have been established. The only filaria parasite that has been used extensively in the laboratory is the rodent filaria *L. sigmodontis*, co-infected with different rodent malaria parasites such as *P. berghei*, *P. chabaudi* and *P. yoelii* in BALB/c and C57BL/6 mice (Graham et al., 2005; Karadjian et al., 2014; Ruiz et al., 2009). Preceding these reports, Yan et al. (1997) used attenuated larvae of *B. pahangi* co-infected with *P. berghei* in CBA/J mice to reveal the immune modulation activity of microfilaremic mice against malaria infection. However, the scanty of studies on *Brugia*-mice models cannot be hidden from the fact that none of the *Brugia* species can complete or develop fully in a mouse. In lieu of these limitations, it necessitates the exploration of new animal models for co-infection of filaria and malaria parasites, in order to increase and broaden the existing knowledge on the interactions that interplay between these parasites. Although *B. pahangi* and *B. malayi* have been established long ago in gerbils (Ash, 1973; Ash & Riley, 1970; Porthouse et al., 2006), and susceptibility of gerbils to *Plasmodium* infections has just been reaffirmed in chapter 3, but the co-infection of these parasites in gerbils has yet to be reported. Hence, the present study used patent *B. pahangi* co-infected with *P. berghei* ANKA, to study the interactions that occur between these two parasites over a time course.

The present study showed that co-infected gerbils have prolonged survival periods than PbA infected gerbils. All gerbils with PbA infections (co-infected gerbils inclusive) died at one point of time or the other due to the malaria challenge, while all the *B. pahangi* infected gerbils survived. Previous report by Specht et al. (2010) has shown that *L. sigmodontis* infection protects against cerebral malaria and prolonged the survival of C57BL/6 mice to subsequent PbA infection. Another study also reported that *L. sigmodontis* infection protects against subsequent *P. berghei* sporozoites infection in BALB/c mice, but the protection could not be replicated in erythrocytic stage of the

malaria parasite (Ruiz et al., 2009). Conversely, concurrent co-infection of gastrointestinal nematode, *H. polygyrus* with self-resolving rodent malaria parasite, *P. chabaudi* AS, in both C57BL/6 and BALB/c mice leads to lower survival rate of co-infected mice while died earlier, whereas when the malaria parasite was co-infected after some days from the pre-existing nematode infection, there were significant increase in the survival rate of the mice (Helmby, 2009). It can then be suggested that nematode infections offer some level of protection against malaria parasites in animal co-infection models.

In the present study, co-infected gerbils showed lower susceptibility and parasitaemia to PbA infections than PbA infected gerbils. Karadjian et al. (2014) have shown that malaria parasitaemia in *P. yoelii* 17XNL infected BALB/c mice was significantly higher than the mice co-infected with *L. sigmodontis*, whereas there was no difference in the malaria parasitaemia in *P. chabaudi* infected mice and those mice co-infected with *L. sigmodontis*. Similarly, previous report showed that *P. berghei* infected C57BL/6 mice had higher significant parasitaemia than mice co-infected with *L. sigmodontis* (Specht et al., 2010). The reports from these studies were in conformity with the present study, and it can be suggested that the outcome of co-infection of filaria and malaria parasite was strongly dependent on the malaria strain but not necessarily the strains of mice or timing of the two infections, either concurrent or successive (Karadjian et al., 2014).

Contrarily, some studies have reported that amicrofilaremic (*L. sigmodontis*) BALB/c mice show higher susceptibility and parasitaemia to both *P. berghei* and *P. chabaudi* AS infection in co-infected mice (Graham et al., 2005; Ruiz et al., 2009). These reports were not in agreement with the preliminary findings in the present study which showed that amicrofilaremic gerbils have lower parasitaemia compared to malaria only and microfilaremic gerbils (Appendix D). The present study did not elaborate or work further

on amicrofilaremic gerbils because post mortem examination on these gerbils revealed that over 80% of the supposed amicrofilaremic, actually have adult worms and/ or microfilariae present at different parts of the gerbil's body or visceral organs. However, the factors responsible for the absence of microfilaria (mf) in the peripheral blood (blood sampled at different times daily for over 21 days post patent period) of gerbils, despite the presence of the parasite in the organs, are yet to be known. Thus, further studies to identify the factors that aid survival and production of mf in vertebrate host are much needed. Though this revelation can be linked to the epidemiological stands of LF, where many exposed individuals are asymptomatic with either mf or no mf, and the few symptomatic individuals bear the severity and burden of the disease.

The disease assessment in this study showed that the temperature, weight gain/loss, haemoglobin and glucose concentrations of gerbils infected with *B. pahangi* infections, were not significantly different from the uninfected control gerbils, and this was expected. Moreover, the reduced body temperature, hypothermia observed in PbA infected gerbils and co-infected gerbils, is a common feature of rodent malaria (Bopp et al., 2010; Cross & Langhorne, 1998; Sanni et al., 2002). The PbA infected and co-infected gerbils, both strongly lost weight and this could also be attributed to the malaria infection, as the filaria infected gerbils gained weight. Hypothermia has been associated with an increase in putative neurotransmitter (5-hydroxytryptamine) turnover, which leads to lower body temperature and food intake (Dascombe & Sidara, 1994). The reduced food and water intake in gerbils infected with PbA (inclusive co-infected gerbils) was the likely cause for the weight loss in these groups of gerbils.

Furthermore, the present study showed that the haemoglobin (Hb) and glucose concentration in the blood of PbA infected gerbils were lower than that of co-infected gerbils. Factors contributing to malaria anaemia have been identified including

phagocytosis of uninfected and infected red blood cells, dyserythropoiesis, erythrocytic suppression, hemozoin which inhibits proliferation of erythroid precursors, and hyper-activated phagocytosis through immunopathogenic mechanisms (Evans et al., 2006; Lamikanra et al., 2007; Thawani et al., 2013). Although helminths such as hookworms, *Ascaris* and schistosomes, have been associated with anaemia (Deribew et al., 2013; Ezeamama et al., 2008; Sangkhathat et al., 2003; Sousa-Figueiredo et al., 2012; Stoltzfus et al., 1997), but there is no substantiated evidence to show that filariasis causes anaemia. Thus, the reduced Hb concentration in the blood of co-infected gerbils can be attributed to the effect of PbA infection. Anaemia in both malaria-only and co-infected mice with filariae has been reported previously (de Souza & Helmby, 2008; Helmby, 2009; Ruiz et al., 2009). Graham et al. (2005) reported that BALB/c co-infected with *L. sigmodontis* and *P. chabaudi* had more severe anaemia than the single malaria infection. However, a study on co-infection of LF and malaria in Malian children and young adults, revealed that filaria infection attenuates immune responses associated with severe malaria and protect against anaemia (Dolo et al., 2012).

Plasmodium has no storage capacity for glycogen, hence depends on exogenous supply of glucose, which is often derived from the vertebrate host. Glucose is an important metabolite for *Plasmodium*, as concentration below 5.5 mM impaires the growth of *P. falciparum in vitro* (Humeida et al., 2011). Hypoglycaemia can be described as increase in glucose use and inhibition of gluconeogenesis, which results in impaired glucose production (Thien et al., 2006). Hypoglycaemia in malaria has also been attributed to the depletion of hepatic glycogen reserves of the host as a result of high metabolic demands for glucose by the parasite, and impairment of gluconeogenesis induced by cytokines (Ogetii et al., 2010; Roe & Pasvol, 2009; White et al., 1983). Previously, hypoglycaemia has been reported in human malaria (Barennes et al., 2005; Murphy & Breman, 2001; White et al., 1987) and rodent malaria (Elased et al., 2001; Elased et al., 1996; Sanni et

al., 2002). However, the present study showed that *B. pahangi* infection protected gerbil against hypoglycaemia during co-infection with PbA. The findings from this study may suggest that the low PbA parasitaemia in co-infected gerbils might not be significant enough to induce hypoglycaemia, as against anaemia observed in PbA infected gerbils. Alternatively, there may be other factors which are responsible for this observation, and this calls for further research to identify the role or presence of *B. pahangi* microfilaria in halting PbA infections to cause hypoglycaemia in co-infected gerbils.

Moreover, it has been hypothesized that in order to resolve a malaria infection, there is need for timely and appropriate immune balance between inflammatory responses required for clearance of the parasite, and anti-inflammatory immune responses needed to prevent immunopathology (Artavanis-Tsakonas et al., 2003; Knowles, 2011; Schofield & Grau, 2005; Walther et al., 2009). Helminths generally are known to induce Th2 immune and T regulatory cell responses. Previous studies have shown that anti-inflammatory responses induced by helminths could hinder the control of *Plasmodium* multiplication, as in the case of helminths increasing the host mortality and hyper-parasitaemia during co-infection with self-resolving *P. chabaudi* and *P. yoelii* infections (Helmby, 2009; Karadjian et al., 2014; Noland et al., 2008). On the other hand, the anti-inflammatory response by helminths could halt immunopathology, as in the case of lethal *P. berghei* co-infection models, helminths tend to reduce mortality and severity associated with malaria such as cerebral malaria (Knowles, 2011; Ruiz et al., 2009; Specht et al., 2010).

The present study shows that co-infected gerbils responded to PbA infection by significant elevation of both proinflammatory and anti-inflammatory cytokines at different time course of infection. Proinflammatory cytokines such as IFN-γ and TNF were elevated at early stage of infection in co-infected gerbils, whereas anti-inflammatory

cytokine, IL-4 was significantly elevated at later stage of infection. The modulatory cytokine, IL-10 is highly expressed by both PbA and co-infected gerbils. Previously, it has been reported that IL-10 was responsible for the suppression of cerebral malaria in co-infection of PbA and *L. sigmodontis* in C57BL/6 mice and this protection was independent of IFN-γ (Specht et al., 2010). Studies have shown that the mechanism underlying this protection is not based on shift in Th1/Th2 balance, as both proinflammatory and anti-inflammatory cytokines are highly produced at the same time or period during course of infection (Babayan et al., 2003; Specht et al., 2010). This hypothesis corresponds to the observation of high elevations of IL-4, IL-6 and IFN-γ at the same time or period in co-infected gerbils, which suggest no switch on immune responses or balance.

Nonetheless, it can be suggested that intrinsic factors such as immune response mechanisms were not enough to determine the interactions that interplay between filaria and malaria parasites during co-infection. Graham (2008) reported that helminths that cause anaemia imposed resources limitations on red blood cells (RBCs)-dependent microparasites such as *Plasmodium*, and thus, reduced the density of the microparasites. Alternatively, anaemia resource limitation as a result of helminth infection may stimulate the host to produce more reticulocytes, and hence increase the replication of *P. berghei* due to its preference for young RBCs (Graham, 2008). The ability of co-infected gerbils in the present study not to succumb to malaria induced-hypoglycemia may serve as an evidence for resource competition between the two parasites. Therefore, it is noteworthy that the present study has shown that there is need for further study to identify the role of extrinsic factors such as competition for space, and competition for nutritional resources such as haemoglobin and glucose between the gerbil host and filaria and malaria parasites.

CHAPTER 5: HISTOPATHOLOGY OF *BRUGIA PAHANGI* AND *PLASMODIUM BERGHEI* ANKA CO-INFECTION IN THE GERBIL (*MERIONES UNGUICULATUS*)

5.1 INTRODUCTION

Malaria and lymphatic filariasis (LF) are co-endemic in many tropical regions (WHO, 2016a; WHO, 2016b), and being mosquito-borne diseases, they can be transmitted by the same vector mosquito (Manguin et al., 2010). Currently, about 3.2 billion people in the world are at risk of having malaria (WHO, 2016a), whereas about 947 million people in 54 countries face the risk of LF (WHO, 2016b). Malaria is more often in the form of acute infection where possible outcomes can either be asymptomatic, uncomplicated or severe. On the other hand, LF is a chronic disease in which the host can harbor the parasite for many years. This may lead to minimal pathology as a result of immunomodulation and tolerance of the host immune system or more severe pathology such as lymphatic inflammation and elephantiasis (McSorley & Maizels, 2012).

Being co-endemic in many areas, co-infections of malaria and filariasis in humans have been reported (Chadee et al., 2003; Muturi et al., 2006; Ravindran et al., 1998). How malaria and filaria parasites interact during co-infection is rarely studied in humans. Many studies have used different nematode parasites (such as *L. sigmodontis*, *H. polygyrus* and *Nippostrongylus brasiliensis*) combination with different rodent malaria parasites, mostly in BALB/c and C57BL/6 mouse strains (de Souza & Helmby, 2008; Graham et al., 2005; Helmby, 2009; Hoeve et al., 2009; Specht et al., 2010), and in non-human primates such as owl monkeys (*Aotus trivirgatus griseimenbra*) (Schmidt & Esslinger, 1981).These studies were embarked on to study the disease outcome in experimental co-infection model. Although these experimental co-infection models mainly analyzed the consequences of filaria infection upon malaria disease outcome, none has studied the histopathological effects/changes on the host except a study by Karadjian et al. (2014),

who used *L. sigmodontis* co-infected with non-lethal strains of *Plasmodium* (*P. yoelii* and *P. chabaudi*) in BALB/c mice to compare their combined effects on the host kidneys and lungs.

Tissue damage due to malaria or filariasis is a common occurrence and need to be abated in order to reduce disease severity. Malaria has been strongly linked to inflammatory responses and its sequestrations in many organs have been attributed to the causes of histopathology in organ-specific manner (Milner et al., 2015). The imbalance immune response to malaria infection is believed to be responsible for inflammations and tissue damage associated with the disease. Nonetheless, glomerulonephritis and chronic kidney damage (Habeeb et al., 2013; Kute et al., 2012a; Taylor-Robinson, 1996; Vuong et al., 1999), hepatic inflammations (Deroost et al., 2014; Whitten et al., 2011), and acute lung injury (Helegbe et al., 2011) have been reported as manifestations of malaria infection.

The pathology of filaria infection can be said to be multifaceted and complex, and often depends on the host and species (Ash & Riley, 1970; Vincent et al., 1980). Although filaria worms are known to be parasite which dwell in the lymphatic systems, but it has been established that the parasite does not only localize within the host heart and lungs, but also reproduce therein (Ash, 1973). Although filariasis mainly causes lymphatic pathology in its hosts, histopathology in other organs have been reported (Dreyer et al., 2000; Mak, 2012).

Nonetheless, both filaria and PbA infections inflict the host with pathology as a result of tissue damage. The present study evaluates the effect of co-infection of *B. pahangi*-PbA on the host tissue, by allowing the filaria infection to be established before co-infection with PbA. Interestingly, previous reports suggest that filaria infections may confer protections against severe malaria (Karadjian et al., 2014; Ruiz et al., 2009; Segura

et al., 2009; Specht et al., 2010; Xiao et al., 1999; Yan et al., 1997). Thus, the present study uses *B. pahangi*, a closely related filaria parasite to *B. malayi*, together with a lethal *Plasmodium* strain (*P. berghei* ANKA) in a gerbil co-infection experimental model, with the aim to assess the pathological effects of co-infection of filariasis and malaria on the animal host. This experiment is a continuum of chapter 4, with the objective of evaluating histopathology of co-infection of *B. pahangi* and *P. berghei* ANKA on gerbil host.

5.2 MATERIALS AND METHODS

5.2.1 Ethical clearance

The study was approved by the Faculty of Medicine Institutional Animal Care and Use Committee (FOM IACUC), University of Malaya, Malaysia (2014/PARA/R/JOQ (Appendix A)).

5.2.2 Sources and maintenance of parasites and gerbils

Gerbils (*Meriones unguiculatus*), were purchased from Charles River (USA) at approximately 4 wks old and kept at the animal facility of the University of Malaya. Gerbils were maintained in individually ventilated cages and supplied with sterilized food and water *ad libitum*. Male gerbils of age 6-8 weeks were used in all experiments in accordance with institutional guidelines for animal care (2014/PARA/R/JOQ). All animals were humanely handled to minimize sufferings.

Brugia pahangi was previously isolated from an infected cat in Carey Island from Klang, Selangor, Malaysia and maintained in gerbils (as described in chapter 4). Infective third-stage larvae (L3) were recovered from *Ae. togoi* mosquitoes at day 11 post infection (pi) as described previously by (Townson, 1997). About 50 L3 in 200 µL phosphate buffered saline (PBS) were inoculated subcutaneously into donor gerbils and allowed for a patency period of about 70 post infection (pi) days.

Plasmodium berghei strain ANKA (MRA-311) was maintained in gerbils via intraperitoneal (ip) inoculation (as described in chapter 4). Briefly, frozen PbA parasitized red blood cells (pRBC) were allowed to thaw at 37^{o}C for 3-5 mins and 0.2 mL was injected into a gerbil to initiate infection. Blood was then harvested by cardiac puncture from the donor gerbil on day 5-7 post infection, and diluted appropriately with phosphate buffer saline (PBS, pH 7.4), before re-introduced into other naive gerbils. Uninfected control animals were given only PBS (pH 7.4).

5.2.3 Experimental infections

The experimental design has been described previously in chapter 4 (Figure 4.2.2). Briefly, the gerbils were divided into four groups. Group 1 was the uninfected control group (N=5) that was given 200 µL PBS subcutaneously (at day 0 and day 70 filaria pi). Group 2 was the filaria-only infected group (N=5), given 50 L3 in 200 µL PBS subcutaneously at day 0 and 200 µL PBS intraperitoneally at day 70 filaria pi. Group 3 was the PbA-only infected group (N=5), given 200 µL PBS subcutaneously at day 0 and 10^6 pRBC in 200 µL PBS intraperitoneally at day 70 filaria pi. Group 4 was the filaria-PbA co-infected group (N=5), given 50 L3 in 200 µL PBS subcutaneously at day 0 and 10^6 pRBC in 200 µL PBS, intraperitoneally, at day 70 filaria pi. The animals were then monitored daily and gerbils were sacrificed (as described in chapter 3) at days 0, 1, 3, 5, 7, 9 and 11.

5.2.4 Histology

Organs including brain, heart, kidney, lung, liver and spleen were removed aseptically from sacrificed animals and fixed in 10% buffered formalin. The spleen and liver of gerbils were assessed morphologically. The spleen index was calculated as ratio of spleen wet weight (g) versus body weight (g) x100 (Specht et al., 2010), whereas the liver index was calculated as ratio of liver wet weight (g) per body weight (g) x100. The tissues were processed using an automated tissue processor (Leica TP1020, USA) and then embedded in paraffin wax. About 3-5 tissue sections (4 µm thick) were randomly cut for both haematoxylin and eosin (H and E) staining and *in situ* hybridization.

5.2.5 *In situ* hybridization

The *in situ* hybridization to detect PbA was performed as described in chapter 3 and Ong et al. (2008). Briefly, 4 µm tissue sections were dewaxed, rehydrated and depigmented with 10% ammonium (70% alcohol, 10 mins). The sections were then

pretreated with 0.1% pepsin (30 mins, 37 ºC), followed by incubation at 95 ºC (10 mins) and 42 ºC (overnight) in standard hybridization buffer together with 1 µL of *Plasmodium* probe (18S ribosomal RNA sequence of *Plasmodium* species Gen Bank M19173.1 and U07368.1). The slides were then subjected to washing and blocking, followed by incubation with anti-digoxigenin-AP Fab fragments (Roche, Switzerland) (1:2000) at 4ºC overnight. The slides were washed and incubated for 2 hrs in liquid permanent red chromogen (Dako, USA) at room temperature. The slides were then counter-stained with Mayer's haematoxylin and mounted with Faramount aqueous mounting medium (Dako, USA).

5.2.6 Modified haematoxylin and eosin staining

The haematoxylin and eosin (H & E) protocol was modified. Briefly, tissue samples were dewaxed in xylene for 5 mins (×3) and hydrated in gradient alcohol concentrations as follows: 100% alcohol for 2mins, 95% alcohol for 2 mins, 95% alcohol for 1 mins, and 95% alcohol for 1 min, respectively. The tissue samples were then stained in 3% Giemsa (Sigma, USA) for 25 mins and washed in running water for 5 mins (×2). Then samples were stained in haematoxylin solution for 3-5 mins and washed in running water for 5 mins. The samples were then stained in eosin solution for 1 min and dehydrated in gradients of alcohol. Samples were allowed to dry before mounting with DPX.

5.2.7 Parasite quantification

The PbA in tissues were quantitated by modifying the methods of Milner et al. (2013) and Seydel et al. (2006). Briefly, each sectioned tissue (Giemsa-H&E stained slides) was assessed microscopically by randomly choosing at least 100 cross-sectioned blood vessels. At least 3 serial sections were examined per organ. Infected RBCs were counted against uninfected RBCs in each blood vessels. The pigmented and unpigmented parasites

within an erythrocyte were included as iRBCs, while extra-erythrocyte malaria pigments were excluded.

5.3 RESULTS

5.3.1 Hepato-splenomegaly caused by *Plasmodium berghei* ANKA infection

The macroscopic morphology of the brain, heart, lungs, kidneys, spleen and liver was examined in all the animal groups. The organs from filaria-infected gerbils appeared normal, except slight enlargement of the spleen (Figure 5.1). The spleen and liver of both PbA-infected and co-infected gerbils were discolored (pigmented) and enlarged (splenomegaly and hepatomegaly respectively), while the lungs of PbA-infected gerbil were more heavily pigmented compared to lungs from co-infected gerbils (Figure 5.1).

The spleen weights to body weight ratio, showed an increase of about 40 folds in PbA-infected gerbils while in co-infected gerbils the increase was relative to about 6 folds observed (Figure 5.2a). Similarly, the liver index of PbA-infected gerbils was significantly ($F_{(6, 48)}$ =34.52, $P < 0.0001$) higher than that of co-infected gerbils, mostly at day 7 and 9, post PbA infection (Figure 5.2b).

Figure 5.1: Morphology of organs harvested from gerbils at different time points.

I. Uninfected control II. Filaria-only infected III. PbA-only infected at day 1 PbA post infection (pi), IV. Co-infected gerbils at day 1 PbA pi V. PbA-only infected at day 9 PbA pi VI. Co-infected gerbils at day 9 PbA pi. The organs are: Br: Brain; H: Heart; Lu: Lungs; Ki: Kidney; Li: Liver; and Sp: Spleen. The spleen of both PbA-only and co-infected gerbils at day 9 PbA pi were grossly enlarged and pigmented. Organs from filaria-only infected gerbils appeared normal except slight enlargement of the spleen.

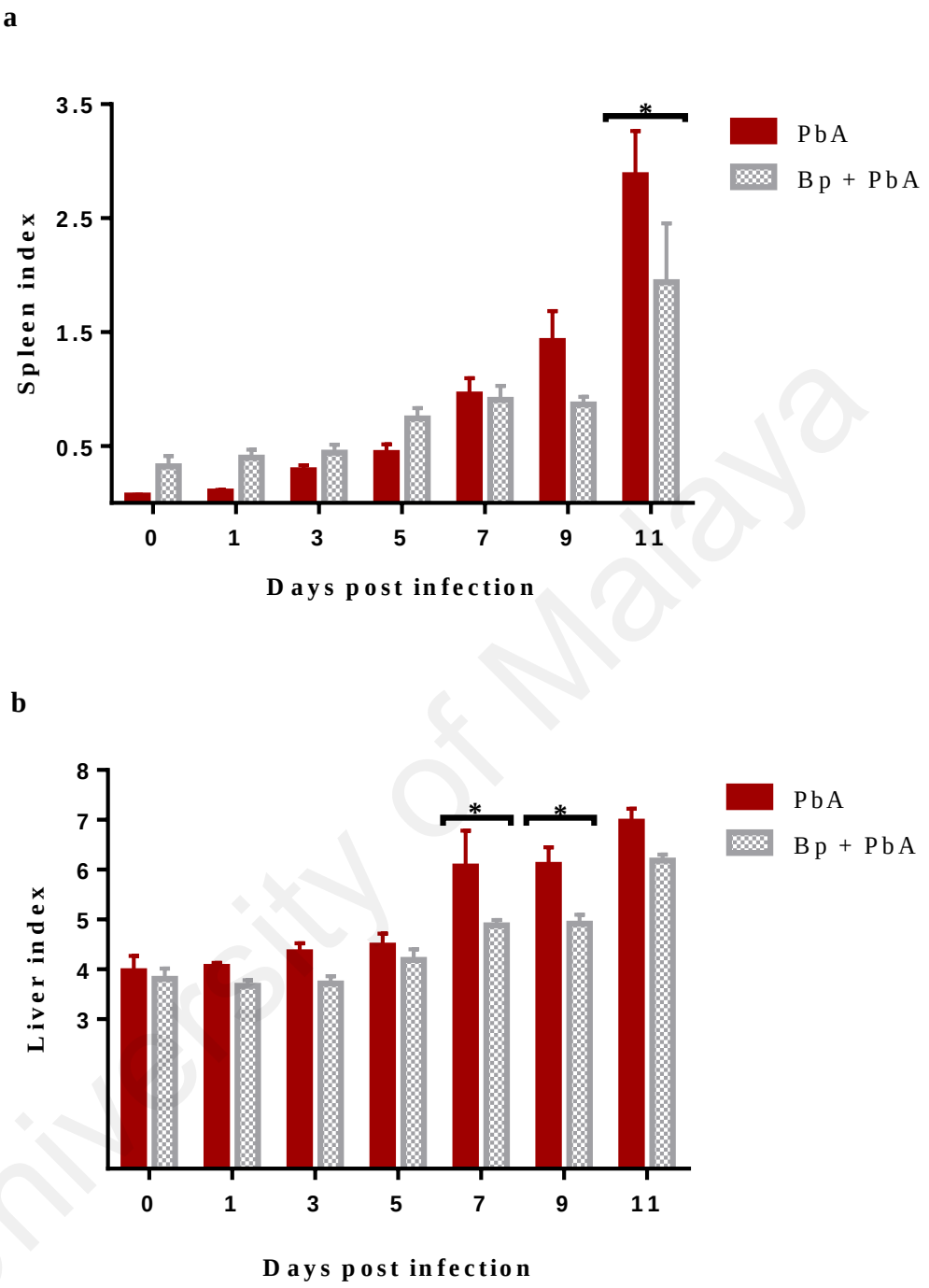

Figure 5.2: Spleen and liver index measured relative to body weight of gerbil over 11-day time course.

a. Spleen index. b. Liver index. Naive gerbils given PBS were included as control and represent day 0 for PbA-single infection, while Bp-single infected gerbils represent day 0 for Bp-PbA co-infection. Bars represent mean ± S.E.M, N = 5. All data were compared by two-way ANOVA with Bonferroni-Sidak's multiple comparison tests for differences between the groups, * $p < 0.05$.

5.3.2 Accumulations of *Plasmodium berghei* ANKA in organs

Aside monitoring of PbA and Bp parasitaemia in peripheral blood (data shown in chapter 4, Figure 4.3), the presence of either or both parasites was examined in various organs. The presence of PbA was confirmed by both *Plasmodium*-genus *in situ* hybridization and Giemsa-H&E methods, while Bp was confirmed with the presence of either microfilaria or adult worm by Giemsa- H&E (Figure 5.3).

Accumulation of infected red blood cells (iRBCs) was monitored in the lungs, kidney and liver. No iRBCs were found at day 1 pi in all organs selected. In the lungs, iRBCs increases steadily from day 3 pi till its peak at day 7 pi in Bp + PbA-gerbils (about 20%) and day 9 pi in PbA-gerbils (about 28%), then declined (Figure 5.4a). There was a significant difference ($F_{(5, 40)}$= 7.740, $P < 0.0001$) in the parasitemia at days 9 and 11 pi in both PbA-gerbils and Bp + PbA-gerbils (Figure 5.4a). Similarly, parasitemia increases in the kidney from day 3 pi until its peak at day 7 pi in PbA-gerbils (about 15%) and Bp + PbA-gerbils (21%), while there was a significant difference ($F_{(5, 40)}$= 7.669, $P < 0.0001$) at day 9 pi (Figure 5.4b). However, there was no significant difference ($F_{(5, 20)}$= 0.0597, $P > 0.05$) in parasitemia found in the liver of both PbA-gerbils and Bp + PbA-gerbils, although the parasitemia was at its peak (about 32%) at day 7 pi in both group of gerbils (Figure 5.4c).

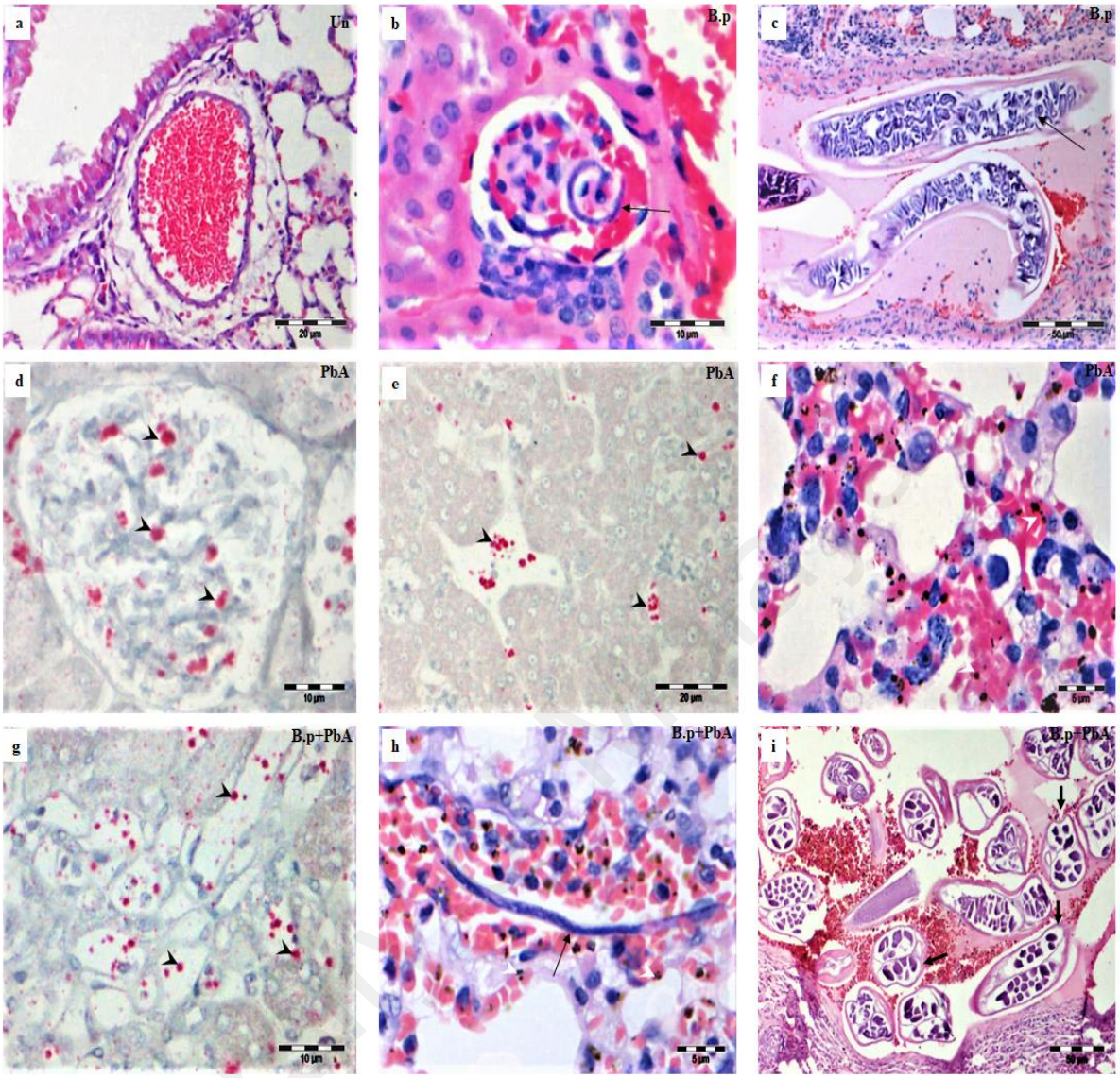

Figure 5.3: Confirmation of PbA and *B. pahangi* in selected organs.

a. lung of an uninfected control gerbil. b. *B. pahangi* microfilaria in kidney of B.p-infected gerbil (thin arrow). c. cross section of adult *B. pahangi* worm (showing developing microfilaria, thin arrow) in the lung of B.p-infected gerbil. d. *P. berghei* ANKA in the glomerulus of PbA-infected gerbil's kidney (black arrow head). e. *P. berghei* ANKA in the liver of PbA-infected gerbil (black arrow head). f. *P. berghei* ANKA and malaria pigments in the lung of PbA-infected gerbil (white arrow head). g. *P. berghei* ANKA in the kidney of B.p+PbA co-infected gerbil (black arrow head). h. *B. pahangi* microfilaria (thin arrow) and malaria pigments (white arrow head) in the lung of B.p+PbA co-infected gerbil. i. cross section of adult *B.pahangi* worm (showing internal organs) and malaria pigments in the lung of B.p+PbA co-infected gerbil. Sections a, b, c, f, h and i were stained with H and E, while sections d, e and g were processed with *Plasmodium* probe-*in situ* hybridization. Bar represents magnification (µm).

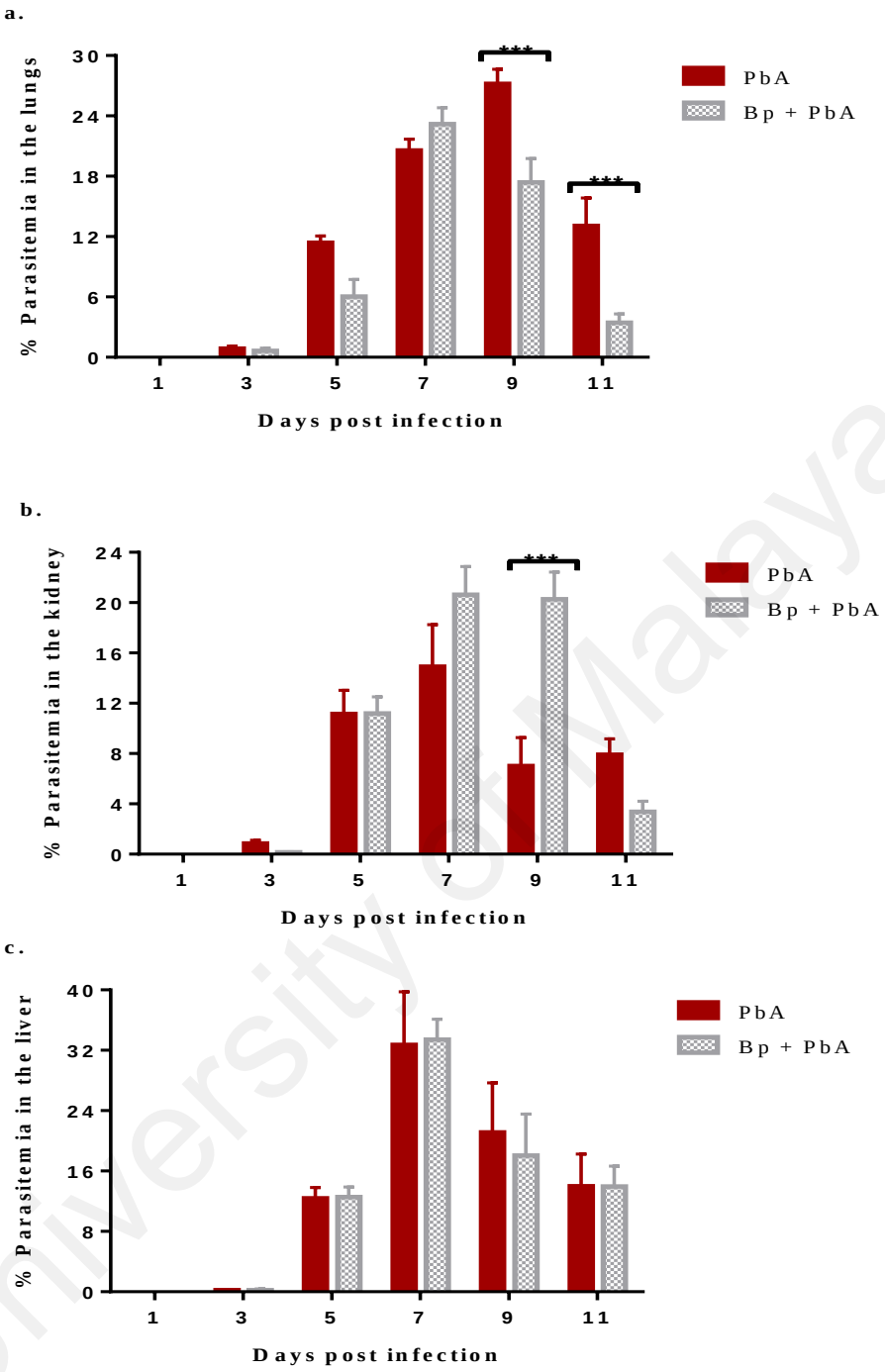

Figure 5.4: Accumulation of PbA iRBCs in blood vessels of the lungs, kidney and liver.

a. lungs b. kidney and c. liver. At least 100 blood vessels from each section of the tissue were observed, and iRBCs were counted against uninfected RBCs. Bars represent mean ± S.E.M, N = 5. All data were compared by two-way ANOVA with Sidak's multiple comparison tests for differences between the groups, *** $p < 0.0001$.

5.3.3 *Brugia pahangi* and *Plasmodium berghei* ANKA infections resulted in pulmonary edema

A histological study was performed to investigate effect of *B. pahangi* and PbA infections on the lungs tissues at different time points. Lungs were harvested at different time points in all the groups and examined for lesions or injuries. No severe lesion other than infiltrations of leucocytes in the lungs of Bp-infected gerbils (Table 5.1). However, lesion such as increase in alveoli cells, leucocytes infiltrations and congestion of cells were commonly observed in both PbA and Bp+PbA co-infected gerbils (Table 5.1). In addition, intrapulmonary hemorrhage congestion was common to PbA infections (Figures 5.5d and g), while filaria granulomas were common to *B. pahangi* infections (Figures 5.5b and i). The bronchi were highly infiltrated and damaged in all the infections (Figures 5.5c, f and i). Hemozoin and PbA were found in the alveoli (Figure 5.5e) while *B. pahangi* microfilaria were trapped in the alveoli as well (Figure 5.5h).

Table 5.1: Pathology observed in the lungs of gerbils

Groups	Time (DPI)	Lesions				
		Increased alveoli cells	Leukocytes infiltrates	Congestion	Haemorrhagic alveolitis	Haemorrhagic bronchitis
UN	7	0	0	0	0	0
Bp	3	0	1/3	0	0	0
	7	0	2/3	0	0	0
	11	0	1/3	0	0	0
PbA	3	1/5	0	0	0	0
	7	5/5	5/5	3/5	5/5	3/5
	11	5/5	5/5	4/5	3/5	3/5
Bp + PbA	3	3/5	4/5	2/5	0	0
	7	5/5	5/5	3/5	2/5	1/5
	11	3/3	3/3	1/3	0	0

The first column shows the four groups of gerbils, second column shows the time of necropsy, while the five sub columns that follows show the lesions observed in the lungs. Values for the lesion indicate: number of gerbils showing the lesion / total number of gerbils studied (n). DPI: Day post infection; UN: Uninfected control gerbils; Bp: *B. pahangi* infected gerbils; PbA: *P. berghei* ANKA infected gerbils; Bp + PbA: gerbils co-infected by *B. pahangi* and *P. berghei* ANKA.

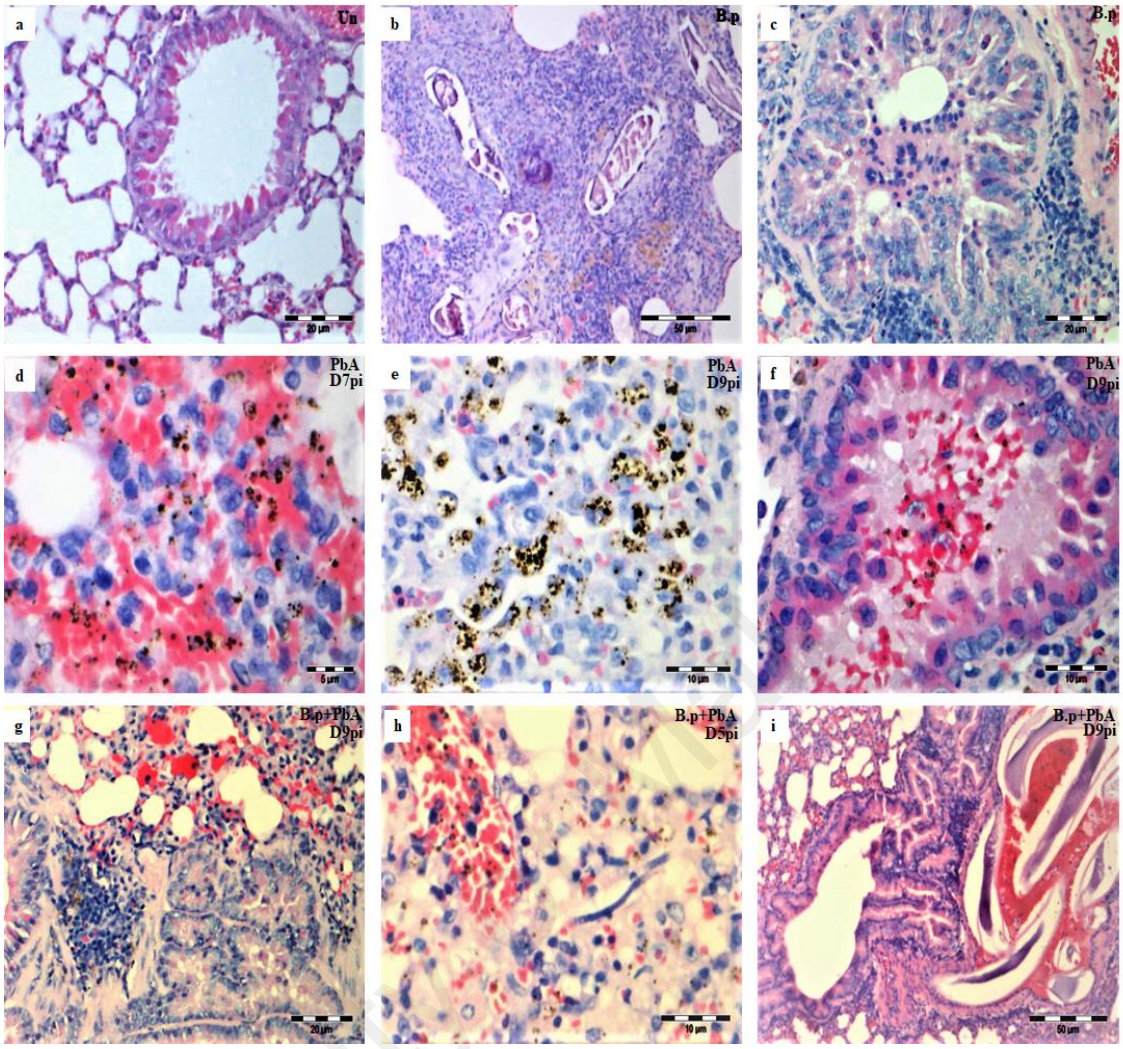

Figure 5.5: Lung injuries during the course of *B. pahangi* and *P. berghei* ANKA infections in gerbils.

a. normal lung in uninfected control gerbils. b. fibrosis and inflammatory reactions from disintegrated worms in B.p-infected gerbil c. inflammatory cells around the bronchus, together with leucocytes infiltrations in B.p-infected gerbil. d. hemorrhage and leucocytes infiltrations in PbA-infected gerbils. e. increased cellular activities and intra-alveolar macrophages with malaria pigments in PbA-infected gerbil. f. distorted bronchus with *P. berghei* ANKA and pigments in PbA-infected gerbil. g. haemorrhage with increased cellular activities in B.p+PbA co-infected gerbil. h. *B. pahangi* microfilaria and *P. berghei* ANKA entrapped in the alveoli of B.p+PbA co-infected gerbil. i. filaria granuloma surrounding dead worms with inflammatory reactions in B.p+PbA co-infected gerbil. All sections were stained with H and E. Bar represents magnification (µm).

5.3.4 Glomerulonephritis associated with *Plasmodium berghei* ANKA infections

The histopathological assessment of the kidney at different time points revealed no histological changes in the kidney of B.p-infected gerbils throughout the course of study (Table 5.2). However, PbA infections caused hemorrhage by day 7 pi and increase in mesangial cells, leucocytes infiltration and damaged glomeruli were all observed in both PbA-infected and B.p+PbA co-infected gerbils by day 11 pi (Figure 5.6).

Table 5.2: Pathology observed in the kidney of gerbils

Groups	Time (DPI)	Lesions		
		Increased mesangial cells	Leukocytes infiltrates	Damaged capillaries
UN	7	0	0	0
Bp	3	0	0	0
	7	0	0	0
	11	0	0	0
PbA	3	2/5	1/5	0
	7	5/5	4/5	3/5
	11	5/5	5/5	4/5
Bp + PbA	3	0	0	0
	7	4/5	3/5	2/5
	11	3/3	2/3	0

First column shows the four groups of gerbils, second column shows the time of necropsy, while the two sub columns that follows show the lesions observed in the kidneys. Values for the lesion indicate: number of gerbils showing the lesion / total number of gerbils studied (n). DPI: Day post infection; UN: Uninfected control gerbils; Bp: *B. pahangi* infected gerbils; PbA: *P. berghei* ANKA infected gerbils; Bp + PbA: gerbils co-infected by *B. pahangi* and *P. berghei* ANKA.

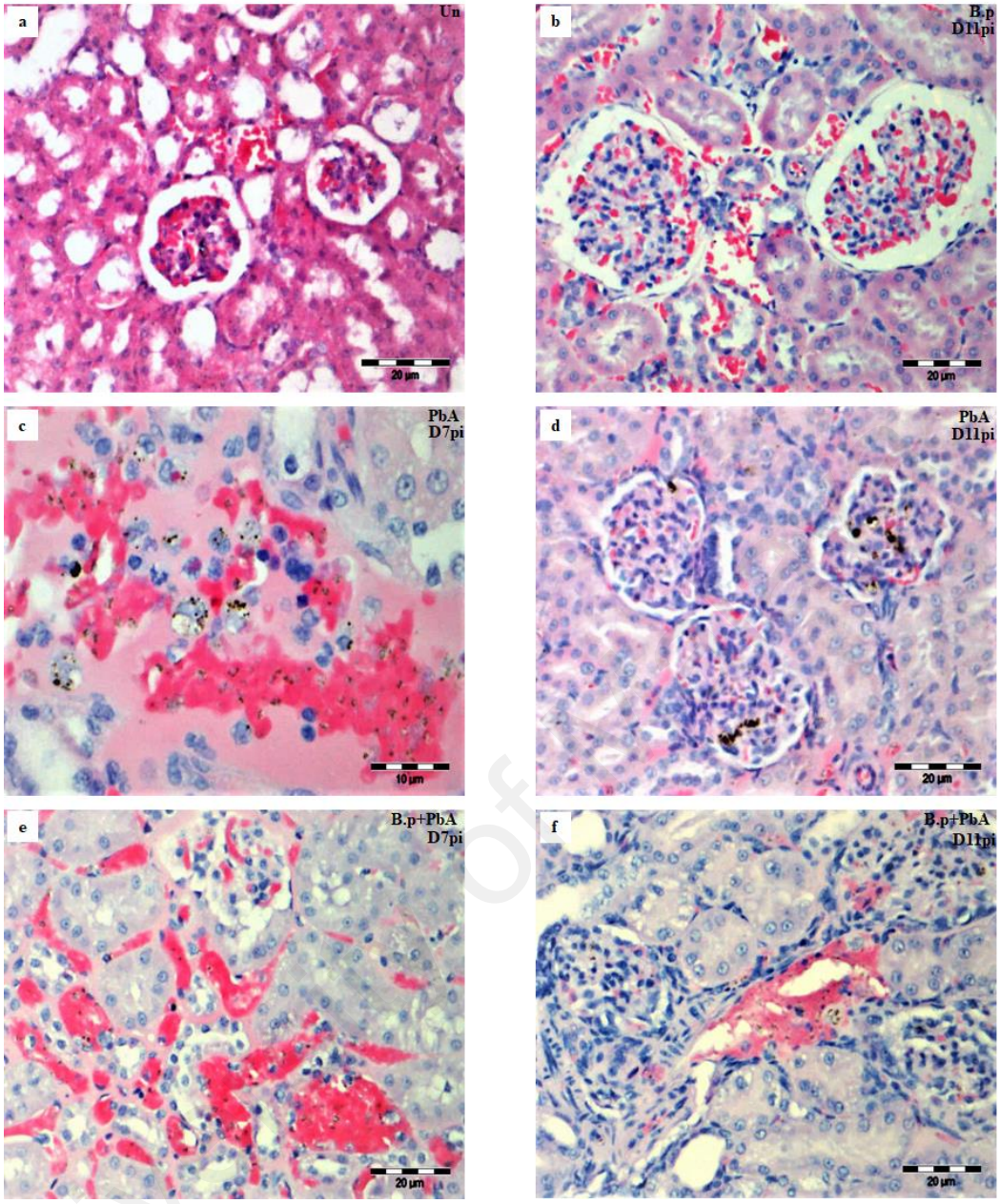

Figure 5.6: Histological changes in the kidney of *B. pahangi* and *P. berghei* ANKA infections in gerbils.

a. Kidney architecture from uninfected control gerbils. b. No histological changes in the kidney of Bp-infected gerbils. c. hemorrhage and infiltrated monocytes in the kidney of PbA-infected gerbils. d. glomerulus with increased mesangial cells and obliterated capillaries in PbA-infected gerbils e. hemorrhage in the kidney of Bp+PbA co-infected gerbils. d. distorted and infiltrated glomerulus with increased mesangial cells in Bp+PbA co-infected gerbils. Bar represents magnification (µm).

5.3.5 *Brugia pahangi* and *Plasmodium berghei* ANKA infections induce extramedullary haematopoiesis

Noticeably in the histological changes during the course of B.p and PbA infections was accelerated hematopoiesis in the liver and spleen (Figure 5.7). In PbA infections, hemozoin deposits were observed in the Kupffer cells (Figures 5.7e and g), while there was congestion of the red pulp in the spleen with hemozoin (Figures 5.7f and h).

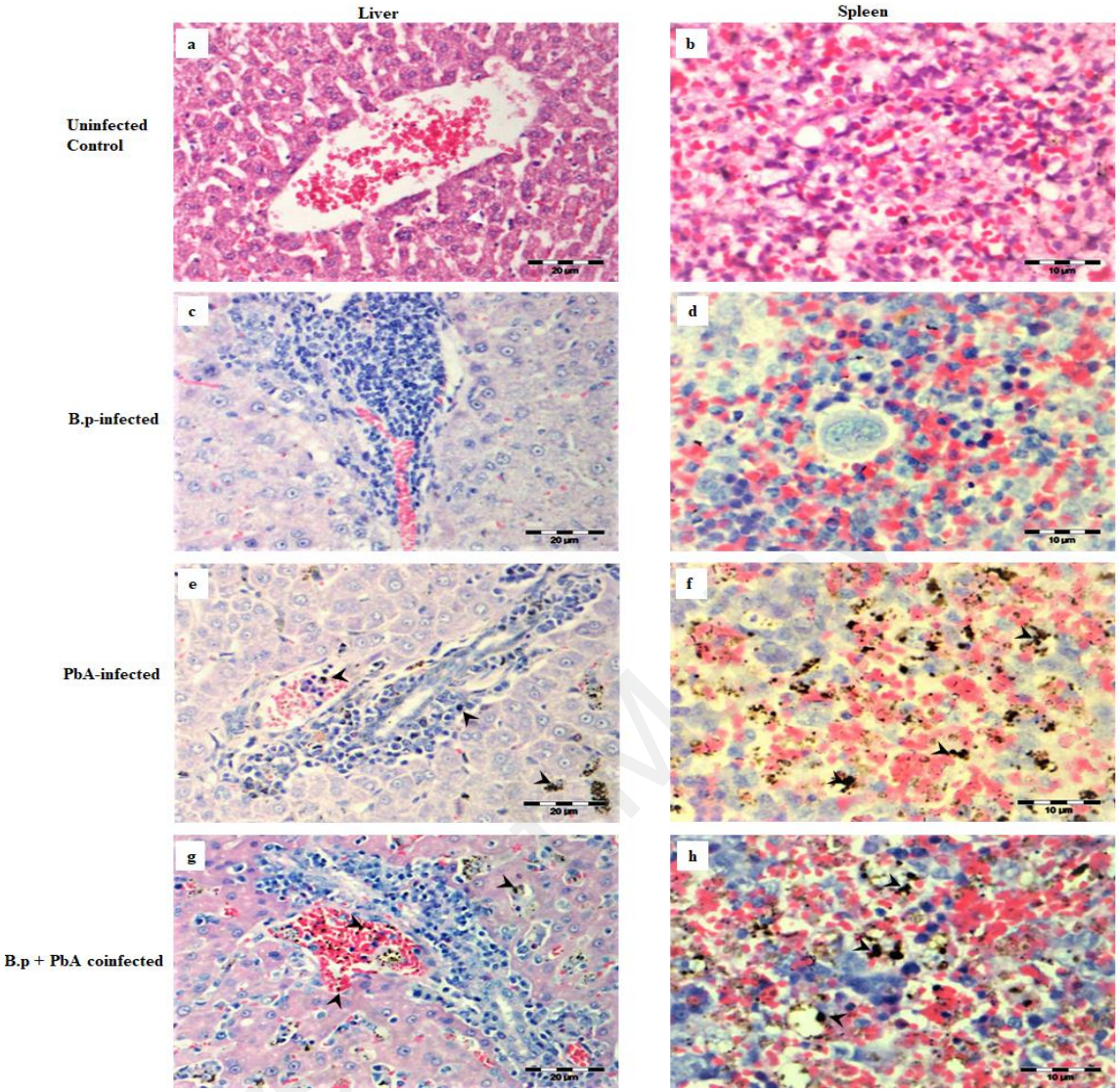

Figure 5.7: Histological changes in the liver and spleen of *B. pahangi* and *P. berghei* ANKA infections in gerbils.

a. normal liver cells structure from uninfected control gerbils. b. normal spleen cells structure from uninfected control gerbils. c. extramedullary hematopoiesis in the liver of B.p-infected gerbils. d. fibrosis surrounded by inflammatory cells in the red pulp of the spleen in B.p-infected gerbils. e. extramedullary hematopoiesis and hemozoin deposits in Kupffer cells from liver of PbA-infected gerbils. f. congestion of red pulp with heavy hemozoin deposits and decreased white pulp content in PbA-infected gerbils. g. congested sinusoids, hemozoin deposits in Kupffer cells, and extramedullary hematopoiesis from the liver of B.p+PbA co-infected gerbils. h. congestion of red pulp with heavy hemozoin deposits in the spleen of B.p+PbA co-infected gerbils. Arrow head show PbA pigments. Bar represents magnification (µm).

5.4 DISCUSSION

The present study examined the histopathology of *B. pahangi*, *P. berghei* ANKA (PbA) and co-infection of *B. pahangi* and *P. berghei* ANKA on gerbil (*Meriones unguiculatus*) hosts. The results presented in this study showed that the pathogenesis of co-infection on gerbils were mostly dependent on PbA infections. Malaria pathology has been ascribed to be due to the ability of malaria parasites to bind to the endothelial cell lining of the brain, lungs, liver and other organs, causing blockage of microvasculature which may result in complicated or severe malaria such as cerebral malaria (CM), severe malaria anaemia, acute lungs injury or acute respiratory distress syndrome (ALI/ARDS), (Lovegrove et al., 2008; Mishra & Newton, 2009; Rogerson et al., 2007). On the other hand, filaria infection mostly affects the lymphatic system and viable adult worms have been identified to cause subclinical lymphangiectasia in human (Dreyer et al., 2000) and animals (Dreyer et al., 1998), though not necessarily through lymphatic obstruction. Lymphatic dysfunction resulting in hydrocele, chyluria, and chylocele are all associated with lymphatic pathology of LF (Dreyer et al., 2000). These lymphatic dysfunctions make the host susceptible to secondary bacterial infections, which is a major cofactor in the progression of the disease into lymphedema and subsequently develop into elephantiasis (Dreyer et al., 2000).

The morphology of selected organs during the course of infection in gerbils showed pigmentation and increase in size of the liver, spleen and lungs in both PbA-gerbils and Bp-PbA co-infected gerbils. These significant weight increases and pigmentation in organs were not observed in Bp-gerbils, suggesting it is due to the PbA infection. Previously, significant increase in spleen index has been reported in both malaria mono infections and malaria co-infections (Karadjian et al., 2014; Ruiz et al., 2009; Specht et al., 2010). In these reports, splenomegaly was more severe in *P. berghei* + *L. sigmodontis* than *P. berghei* only (Specht et al., 2010), though no difference was observed in similar

parasites combination by Ruiz et al. (2009), while combinations of *P. yoelii* + *L. sigmodontis* and *P. chabaudi* + *L. sigmodontis* also showed more increase in spleen index than their respective *P. yoelii* and *P. chabaudi* mono infections (Karadjian et al., 2014). However, the extent of splenomegaly reported in these studies (about 10 folds) is lower in comparison with the present study (about 30 folds), which shows higher splenomegaly in PbA-gerbils than Bp-PbA co-infected gerbils. Co-infection of *P. yoelii* and *Schistosoma mansoni* has also been reported to show higher liver and spleen mass than *P. yoelii* mono-infection (Sangweme et al., 2009).

The spleen serves a key role during malaria infection, where it helps in removal of damaged and iRBCs, stimulates the immune response and also aids in production of new RBCs (Engwerda et al., 2005). The removal of both damaged and iRBs from circulation by the spleen contributes to its heavy pigmentation during malaria infections (Angus et al., 1997; Chotivanich et al., 2002). As a result, accumulations of hemozoin on the tissue may contributes to increase in the organ's weight. Splenomegaly and hepatomegaly were observed to be increasing progressively during the course of malaria infections in gerbils. This has been suggested to be a result of activities of macrophages and dendritic cells in these organs, which help to capture antigen for the generation of acquired immune responses (Engwerda et al., 2005), thereby producing and recruiting more cells to the infection sites.

Furthermore, the ability of iRBCs to sequester in organs, hinder the spleen in removing iRBCs from circulation and thus, aids parasites survival and maintenance (Buffet et al., 2011; Fonager et al., 2012). So, the adherence of iRBCs to endothelia cells has been attributed to the cause of severe malaria pathogenesis (Hisaeda et al., 2005). The present study showed that patent *B. pahangi* infection could not stop accumulation of PbA iRBCs in selected organs, although more parasites were encountered in PbA-gerbil than Bp-PbA

co-infected gerbils. However, it has been proposed that the accumulation of malaria parasites in the microvessels of organs will mechanically block the blood vessels (Miller et al., 2002). Due to the ability of the parasite to cause anaemia (Schofield, 2007), it may lead to shortage of blood supplies and invariably organ failure. In the present study, the parasitaemia increased steadily before it started to drop from day 7 post PbA infection, which coincided with the peak period of anaemia (Figure 4.5a) in PbA infected gerbils.

Among organs that showed tissue damage during the course of filaria and malaria infections include the lungs. During malaria infection, the binding of iRBCs to the pulmonary microvasculature of the lungs, activates the endothelium and leukocytes, thereby activating the release of cytokines and up-regulation of adhesion molecules (Frevert et al., 2014). This results in the accumulation of monocytes and the aftermath effects lead to malaria-associated ALI/ARDS. In human malaria infection, there have been reported cases of ALI/ARDS associated with *P. falciparum* (Genrich et al., 2007; Maguire et al., 2005), *P. vivax* (Anstey et al., 2007; Tan et al., 2008; Valecha et al., 2009) and *P. ovale* (Lee & Maguire, 1999; Rojo-Marcos et al., 2008). Also, several murine model studies have been used to demonstrate the pathogenesis of malaria-associated ALI/ARDS (Deroost et al., 2013; Hee et al., 2011; Helegbe et al., 2011; Lovegrove et al., 2008).

The presence of mf and adult *B. pahangi* in the lungs or pulmonary circulation of gerbils is not surprising, as it has been established that developing filaria infective larvae (L3) undergo intralymphatic migration, through the host body subcutaneously via the lymphatics to their localizing niche, mostly the lymphatic system, cardiopulmonary system, connective tissues or serous cavity (Allen et al., 2008; Babayan et al., 2003; Bain et al., 1994; Karadjian et al., 2017). In addition, the migrating young adults and

microfilariae in the lungs have been identified to result in pulmonary infiltrates and eosinophilia (Dreyer et al., 1996; Magnussen et al., 1995; Rocha et al., 1995).

Nonetheless, the present study has demonstrated that both adult worm and mf of *B. pahangi* dwell in the lungs of gerbils. The underlying pulmonary granulomas associated with *B. pahangi* infection could not be reverse in the presence of malaria co-infection. The damage or injury to the lungs in co-infected gerbils appeared to be more severe, partly due to the existing leucocyte infiltrations and granulomatous inflammations afflicted by the filaria infection and combined with the haemorrhagic alveolitis and bronchi due to malaria infection. However, the observations here differ with study by Karadjian et al. (2014), which reported leucocyte infiltration in the lungs of *L. sigmodontis*-infected mice only but affirmed that the filaria worm plays no protective role in the mild lung injury observed in co-infected mice. Hence, the present study shows that *B. pahangi* plays no protective role on the lesions observed in the lungs of co-infected gerbils and that, both PbA and B.p-PbA co-infected gerbils could have suffered from severe injury to the lungs during course of PbA infections.

There are serious concerns on the histopathology of malaria and filaria infection, as pathology of both diseases have been associated with renal injury in human studies (Dreyer et al., 1999a; Dreyer et al., 1992; Kute et al., 2012b; Sinha et al., 2013). A study by Nacher et al. (2001) has shown that malaria patient with acute renal failure had increased liver abnormalities and this may not be necessarily associated with the cytoadherence properties of the malaria parasite, unlike cerebral malaria. An animal study showed that PbA infection does not result in histological damage or injury on the kidneys of both Balb/c and CBA mice (Helegbe et al., 2011). Conversely, another study has revealed the sequestration of *P. yoelii* 17XL in the kidney of Balb/c mice, while *P. yoelii* 17NXL could not sequester or parasitize the microvessels of the kidney of the same

mouse strain (Fu et al., 2012). However, the present study showed the presence of both *B. pahangi* microfilaria and PbA in the microvessels and glomeruli of gerbil's kidney. The findings from chapter 3 have shown that there is no evidence of cytoadherence of PbA in any of selected gerbil's tissue or organs. Nevertheless, the possibilities of renal damage or failure cannot be excluded.

The inflammation observed in the gerbil's kidney in this study, can be attributed to PbA infection. Although, mf was seen in blood vessels of the kidney of *B. pahangi* infected gerbils, but no damage or inflammation was observed. Glomerulonephritis, proteinuria and hematuria have been reported previously to be associated with *W. bancrofti* infection (Dreyer et al., 1999a). Although the real cause of renal damage due to filaria infection is yet to be known. It has been suggested that circulating mf may be responsible for mechanical damage done to the glomeruli (which results in haematuria), while inflammation as a result of immune response has been identified as potential cause as well (Dreyer et al., 1999a). Similarly, renal damage due to bancroftian filariasis has been reported previously in 30% asymptomatic male microfilaremic carriers (Dreyer et al., 1992). Circulating mf have been identified to be the major cause of tissue damage during extra-lymphatic filariasis, but this could not be proven in the present study. Contrary to these previous studies, proteinuria and hematuria are not determined in the present study and thus, this call for further study to identify more diagnostic marker associated with histopathology of the kidney during co-infection of *B. pahangi* and PbA in gerbils. However, the present study is in contrast to the report of Karadjian et al. (2014), where *L. sigmodontis* showed a protective effect on lesions in the Balb/c kidney, when co-infected with either *P. yoelii* or *P. chaubadi*. Here, *B. pahangi* infection showed no protective effect on gerbil's kidney when co-infected with *P. berghei* ANKA.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

Overall, the present study has shown that gerbils can serve as a good model for severe malaria and co-infection of brugian filariasis and malaria. *Brugia pahangi* infection protects against malaria clinical manifestations such as hypoglycaemia and severe anaemia, and also prolong the lifespan of the gerbils. The outcome of this research is in corroboration with previous studies that showed filaria infection protects against severe malaria in both human and animal studies.

The establishment of PbA infections in gerbils show that gerbils are highly susceptible to malaria infections and as low as 100 PbA parasites was enough to initiate infections. Clinical symptoms such as ruffled hair, hunchback and wobbly gait were experienced by gerbils during PbA infections. Pathological symptoms such as weight loss, hypothermia, anaemia, splenomegaly and hepatomegaly were also found to be associated with PbA infections in gerbils. In addition, gerbil immune response to PbA showed the production of both pro-inflammatory cytokines (IFN-γ and TNF) and an immune-modulatory cytokine (IL-10).

Furthermore, the present study showed that there were interactions that occurred within the gerbil host, during co-infection of PbA and *B. pahangi*. There was hypothermia and weight loss being experienced by both PbA-infected and co-infected gerbils, apparently due to significant loss of appetite for food and water. More importantly, co-infected gerbils survived longer than PbA infected gerbils. However, co-infected gerbils do not show pathological signs of hypoglycemia and severe anaemia as shown by PbA infected gerbils. Similarly, co-infected gerbils were less susceptible to PbA infections. Co-infected gerbils responded early to PbA infection by elevation of both proinflammatory and anti-inflammatory cytokines during the course of infections.

Moreover, the present study has been able to show the evidence on the accumulation of iRBCs in different organs of the host. More importantly, it is speculated that PbA to have sequestered in the organs, as observed in *in situ* hybridization and modified H & E staining. Histopathological changes in multiple organs of *B. pahangi*-infected and PbA co-infected gerbils are mostly induced by PbA infections except for the acute lungs injury which both parasite (individually) contributed to the severe damage. Notably, hepatosplenomegaly was observed among PbA-infected and co-infected gerbil (majorly induced by PbA infections), and leucocytes infiltrations, filaria granuloma and hemorrhagic alveolitis were observed in the lungs of co-infected gerbils. Also, the inflammations and damage to the glomeruli of gerbil's kidney, and increased hemopoietic activities of the spleen and liver associated with PbA infections, are of great importance.

It is noteworthy that in the context of human infections, there are limitations in extrapolating the findings from animal co-infections studies. In Africa, the timing of malaria and filaria assaults on individuals takes place on different time scales and initial infection intensifies over months or years. Malaria infections in children often resulting in severe life-threatening malaria initially, before chronic and relapsing malaria becomes norm; while filaria may be only acquired as a more chronic condition as the individual ages. Peak prevalence of filaria is around 20 years old age groups, with many years of initial infections display of pathology.

6.2 RECOMMENDATIONS

There have been intensified efforts to eliminate LF in the world, with the use of MDA to champion the course. Considering the outcome of the present study and evidences from other studies in both human and animals, elimination of LF will surely aid or influence transmission of malaria, since both parasites are being transmitted by the same vector hosts in endemic areas. In lieu of this, there is an urgent need to evaluate the role or effect

of MDA in the transmission of malaria. This can be simulated in the laboratory by assessing the susceptibility of gerbils treated for filariasis, to malaria infection. This can be evaluated further by assessing the possibility of concomitant transmission of filaria and malaria parasites in gerbil host to vector host and compared to the transmission from treated gerbil host. Though these may be challenging, as it is a huge task to establish a concomitant infection in mosquitoes from co-infected animal host in the laboratory. Our trials on *Anopheles dirus ss* seems promising, as it picks up mf from infected gerbil and develop to infective larval stage for the *B. pahangi* and also, both oocysts and sporozoites were observed when infected with *P. berghei* ANKA.

Furthermore, the present study has established the co-infection of filaria and malaria parasite in the gerbil model and highlighted the interactions that occurred within the gerbil host. However, there is a need to further evaluate the effect of the parasites on each other in co-infection states. Notably, these effects may be due to the host immune pressure or other intrinsic factors, and thus can be revealed by analysing the gene expression of each parasite in mono-infection and co-infection states. This will also reveal novel or unique genes that are preferentially expressed in each condition.

In addition, there is a need to further establish the suitability of gerbil to other plasmodia infections such as non-lethal, *P. chabaudi* and also, its tolerability to murine filaria, *L. sigmodontis*.

6.3 LIMITATIONS

The most challenging aspect in this study was the propagation of *B. pahangi*, as it requires a suitable vertebrate host and efficient vector host. The complete cycle of *B. pahangi* requires about 90 or more days, and quite a number of mf are required to continue propagation. Occasionally, not all gerbils become positive after the patency period. In lieu of these challenges, we embarked on cryopreservation of the mf, but all were not

successful. There are yet to be a breakthrough in the *in vitro* culturing of *B. pahangi* from adult to mf production.

Mixed infections of *B. pahangi* and PbA in mosquitoes were part of the objectives considered initially for this study. After several attempts, PbA infection was not successful in *Ae. togoi* and several efforts were also made on PbA infections in *An. cracens* or *B. pahangi* infections in *An. cracens*, but all to no success. However, *An. dirus* was later discovered to be susceptible to *B. pahangi* and PbA mono infections. Considering the tasks of establishing colonies of *An. dirus ss* in the laboratory, coupled with time constraints, the objective was dropped.

Other limitations include the lack of gene knock-out gerbils to further explore the roles of the cytokines in this study. Also, there is a constraint in the ability to quantify gerbil cytokine protein levels, as there are no kits available commercially.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Paper I: **Junaid, Q. O.**, Khaw, L. T., Mahmud, R., Ong, K. C., Lau, Y. L., Borade, P. U., Liew, K. W., Sivanandam, S., Wong, K. T., Vythilingam, I. (2017). Pathogenesis of *Plasmodium berghei* ANKA infection in the gerbil (*Meriones unguiculatus*) as an experimental model for severe malaria. *Parasite, 24*(38).

Paper II: **Junaid, Q. O.**, Mahmud, R., Khaw, L. T., Sivanandam, S., Vythilingam, I. (2018). *Brugia pahangi* co-infection with *Plasmodium berghei* ANKA in gerbils protect against severe malaria. *Manuscript submitted.*

Paper III: **Junaid, Q. O**., Wong, K. T., Mahmud, R., Khaw, L. T., Ong, K. C., Vythilingam, I. (2018). Histopathology of *Brugia pahangi* and *Plasmodium berghei* ANKA co-infection in the gerbil (*Meriones unguiculatus*). *Manuscript submitted.*

Publication not related to the research

Al-Abd, N. M., Nor, Z. M., **Junaid, Q. O.**, Mansor, M., Hasan, M. S., & Kassim, M. (2017). Antifilaria activity of caffeic acid phenethyl ester on *Brugia pahangi in vitro* and *in vivo*. *Pathogens and Global Health, 111*(7), 388-394.

Conference

Junaid, Q. O., Khaw, L. T., Mahmud, Wong, K. T., R., Ong, K. C., Sivanandam, S., Vythilingam, I. (2016). Gerbil (*Meriones unguiculatus*): An experimental model for Malaria. Presented at *Molecular Approach to Malaria 2016*, held at Mantra Lorne, Victoria, Australia, on 21st – 25th February, 2016.