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Histopathology of *Brugia pahangi* and *Plasmodium berghei* ANKA co-infection in the Gerbil (*Meriones unguiculatus*)

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Abstract. Co-infection with multiple different parasites is a common phenomenon in both human and animals. Among parasites that frequently co-infect the same hosts, are the filarial worms and malaria parasites. Despite this, the mechanisms underlying the interactions between these parasites is still relatively unexplored with very few studies available on the resulting pathologies due to co-infection by filarial nematodes and malaria parasites. Hence, this study investigated the histopathological effect of *Brugia pahangi* and *Plasmodium berghei* ANKA (PbA) infections in gerbil host. Gerbils grouped into *B. pahangi*-infected, PbA-infected, *B. pahangi* and PbA-coinfected, and uninfected control, were necropsied at different time points of post PbA infections. *Brugia pahangi* infections in the gerbils were first initiated by subcutaneous inoculation of 50 infective larvae, while PbA infections were done by intraperitoneal injection of 10^6 parasitized red blood cells after 70 days patent period of *B. pahangi*. Organs such as the lungs, kidneys, spleen, heart and liver were harvested aseptically at the point of necropsy. There was significant hepatosplenomegaly observed in both PbA-infected only and coinfected gerbils. The spleen, liver and lungs were heavily pigmented. Both *B. pahangi* and PbA infections (mono and co-infections) resulted in pulmonary edema, while glomerulonephritis was associated with PbA infections. The presence of both parasites induced extramedullary hematopoiesis in the spleen and liver. These findings suggest that the pathologies associated with coinfected gerbils were synergistically induced by both *B. pahangi* and PbA infections.

INTRODUCTION

Malaria and lymphatic filariasis (LF) are co-endemic in many tropical regions (WHO, 2016a, 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, 2016b), whereas about 947 million people in 54 countries face the risk of LF (WHO, 2016a). Malaria is more often manifested in the form 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 filaria parasites in humans have been reported (Kwan *et al.*, 2018; M'bondoukwé *et al.*, 2018; Muturi *et al.*, 2006; Stensgaard *et al.*, 2016). Furthermore, some studies have been conducted on co-infection of *Litomosoides sigmodontis* with different rodent malaria parasites, mostly in BALB/c and C57BL/6 mouse strains (Graham *et al.*, 2005; Specht *et al.*, 2010) and in non-human primates such as owl monkeys (*Aotus trivirgatus griseimenbra*) (Schmidt & Esslinger, 1981). These experiments were conducted to study the disease outcome in experimental co-infection model. Although these experimental co-infection models mainly analyzed the consequences of filarial infection upon malaria disease outcome, none had studied the resulting histopathological effects/changes on the host with the exception of 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 as a result of 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 have been attributed to be the causes of histopathology (Milner *et al.*, 2015). The imbalanced 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 also been reported as manifestations of malaria infection.

The pathology of filarial infection, on the other hand, is said to be multifaceted and complex, depending on the host and species (Ash & Riley, 1970; Vincent *et al.*, 1980). Although filarial worms are known to dwell in the lymphatic systems, it has been established that the parasite localize and reproduce within the host heart and lungs (Ash, 1973). Although filariasis mainly causes lymphatic pathology in its hosts, pathologies in other organs have also been reported (Dreyer *et al.*, 2000; Mak, 2012).

Filarial infections inflict injury on the host through larva and young adult migration, while PbA infections cause pathology to the host through sequestrations and tissue damage. Hence, it can be hypothesized that combined pathological effect of co-infection of filarial and PbA in a single host will be more severe than mono infections. The present study evaluates the effect of co-infection of *B. pahangi*-PbA on the host tissues, by allowing the filarial infection to be established before co-infection with PbA. Interestingly, previous reports suggest that filarial infections may confer protections against severe malaria infection (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 filarial parasite to *B. malayi*, together with a lethal *Plasmodium* strain (PbA) in a gerbil co-infection experimental model with the aim to assess the combined pathological effects of filaria and malaria on the animal host.

MATERIALS AND METHODS

Ethical approval

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).

Sources and maintenance of parasites and gerbils

Gerbils (*Meriones unguiculatus*), were purchased from Charles River (USA) at approximately 4 weeks 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, Selangor, Malaysia, and maintained in gerbils. Infective third-stage larvae (L3) were recovered from *Aedes 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 pi days.

Plasmodium berghei strain ANKA (MRA-311, USA) was maintained in gerbils via intraperitoneal (ip) inoculation. Briefly, frozen PbA parasitized red blood cells (pRBC) were allowed to thaw at 37°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 pi, and diluted appropriately with PBS (pH 7.4), before re-introduction into other naive gerbils. Uninfected control animals were given only PBS (pH 7.4).

Experimental infections

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 filarial pi). Group 2 was the filarial-only infected group (n=15), given 50 L3 in 200 µL PBS subcutaneously at day 0 and 200 µL PBS intraperitoneally at day 70 filarial pi. Group 3 was the PbA-only infected group (n=30), given 200 µL PBS subcutaneously at day 0 and 10^6 pRBC in 200 µL PBS intraperitoneally at day 70 filarial pi. Group 4 was the filarial-PbA co-infected group (n=30), given 50 L3 in 200 µL PBS subcutaneously at day 0 and 10^6 pRBC in 200 µL PBS, intraperitoneally, at day 70 filarial pi. The gerbils were euthanized with overdose of ketamine (Troy Laboratories, Australia) and xylazine (Santa Cruz Animal Health, USA) at days 0, 1, 3, 5, 7, 9 and 11, for histopathology studies.

Histopathology

Organs including brain, heart, kidneys, lungs, 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. Chronological histopathology on the lungs and kidneys were characterized according to methods of (Karadjian *et al.*, 2014).

***In situ* hybridization**

The *in situ* hybridization to detect PbA was performed as described by Ong *et al.* (2008) with some modifications. 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°C), followed by incubation at 95°C (10 mins) and 42°C (overnight) in standard hybridization buffer together with 1 µL of *Plasmodium* probe (curtesy of Dr. Lau Yee Ling) (Junaid *et al.*, 2017). The slides were then subjected to washing and blocking, followed by incubation with anti-digoxigenin-AP Fab fragments (Roche, Switzerland) (1:2000) at 4°C 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).

Modified haematoxylin and eosin staining

A modification of the haematoxylin and eosin (H & E) staining protocol was employed. Briefly, tissue samples were dewaxed in xylene for 5 mins (×3) and hydrated in gradient alcohol concentrations as follows: 100% for 2 mins, 95% for 2 mins, 95% for 1 mins, and 95% for 1 min, respectively. The tissue samples were then stained in 3% Giemsa (Sigma, USA) for 25 mins and washed in tap 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.

Parasite quantitation

The PbA in tissues were quantitated by the methods of Milner *et al.* (2013); Seydel *et al.* (2006) with some modifications. Briefly, each sectioned tissue was stained with Giemsa-H&E and assessed microscopically by randomly choosing at least 100 cross-sectioned blood vessels. At least, 3 serial sections of the tissue block were examined per organ. Infected RBCs were counted against uninfected RBCs in each blood vessels, to determine the parasitaemia level. The pigmented and unpigmented parasites within an erythrocyte were included as parasitized RBCs, while extra-erythrocyte malaria pigments were excluded.

Statistical analysis

All data were analysed using GraphPad prism 6.0 (San Diego, CA, USA). The distribution of the data was assessed by a Kolmogorov-Smirnov test for normality testing. Two-way ANOVA was used to analyse all data and Bonferroni-Sidak's multiple comparison test was used for post-hoc test to determine differences between groups. All results are expressed as mean ± S.E.M (Standard Error of Mean) and considered statistically significant when $p < 0.05$.

RESULTS

Hepato-splenomegaly caused by PbA infection

The macroscopic morphology of the brain, heart, lungs, kidneys, spleen and liver, were examined in all the animal groups. The organs from filarial-infected gerbils appeared normal, except slight enlargement of the spleen (Fig. 1). The spleen and liver of both PbA-infected and coinfected 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 coinfected gerbils (Fig. 1).

The spleen weight to body weight ratio of *B. pahangi* infected gerbil increased by 5 fold, but was not significantly ($F_{(6, 96)}=1.14$, $P = 0.3313$) different from spleen index (SI) of uninfected gerbils (Fig. 2a). The SI of PbA infected gerbil progressively increased over 40-fold from D0 to D11 pi and was significantly ($F_{(6, 96)}=46.10$, $P < 0.0001$) higher than the SI of *B. pahangi* and co-infected gerbils at days 9 and 11 pi. Similarly, Fig. 2b shows that the liver index (LI) of PbA infected gerbils steadily increased from 3.9 to 6.9 at days 0 to 11 pi and it was significantly higher ($F_{(6, 96)}=38.01$, $P < 0.0001$) than LI of *B. pahangi* and co-infected gerbils from days 7 to 11 pi.

Presence and accumulations of *Plasmodium berghei* ANKA in organs

The presence of either or both parasites were examined in various organs. The presence of PbA was confirmed by both *Plasmodium*-genus *in situ* hybridization and giemsa-H&E methods, while *B. pahangi* was confirmed with the presence of either microfilaria or adult worm by Giemsa- H&E (Fig. 3).

Accumulation of pRBCs was monitored in the lungs, kidney and liver. There was significantly ($F_{(5, 40)}=7.740$, $P < 0.0001$) higher PbA pRBCs in the lungs of PbA-infected gerbils than B.p + PbA-gerbils at days 9 and 11 pi (Fig. 4a). Conversely, PbA parasitemia was significantly ($F_{(5, 40)}=$

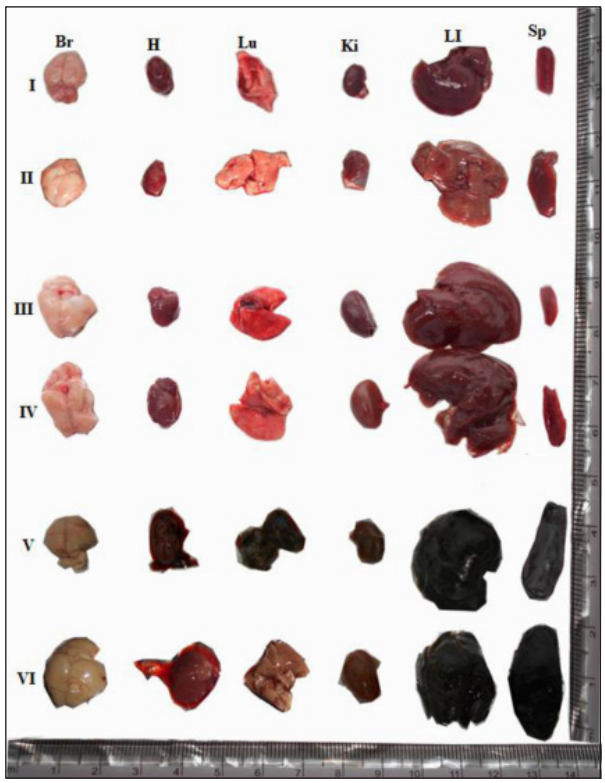

Figure 1. Morphology of organs harvested from gerbils at different time points. I. Uninfected control II. Filarial-only infected III. PbA-only infected at day 1 PbA post infection (pi), IV. Coinfected gerbils at day 1 PbA pi V. PbA-only infected at day 9 PbA pi VI. Coinfected gerbils at day 9 PbA pi. The organs are: Br: Brain; H: Heart; Lu: Lungs; Ki: Kidney; Li: Liver; and Sp: Spleen. The spleen of both PbA-only and coinfected gerbils at day 9 PbA pi are grossly enlarged and pigmented. Organs from filarial-only infected gerbils appeared normal except slight enlargement of the spleen.

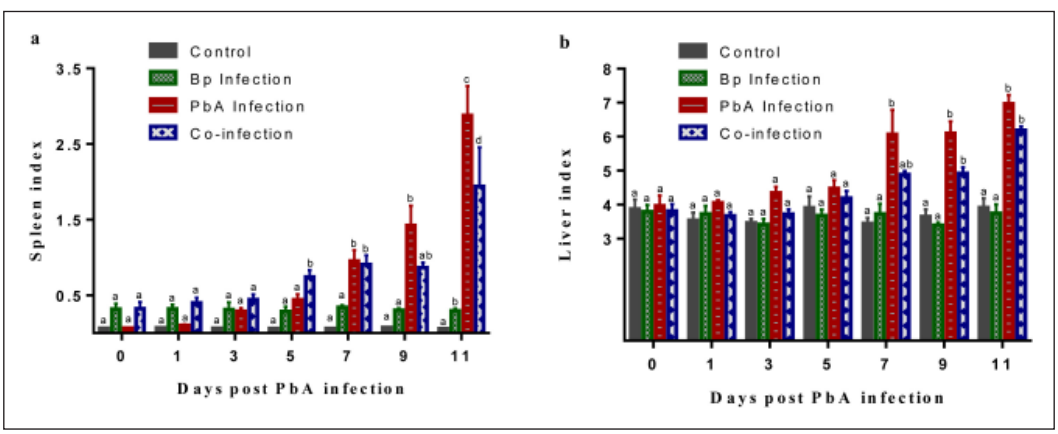

Figure 2. Spleen and liver index measured relative to body weight of gerbil over 11-day time course. a. Spleen index. b. Liver index. The control gerbils are the uninfected gerbils injected with PBS, Bp infection is a patent *B. pahangi* infection, PbA infection is a *Plasmodium berghei* ANKA infection while co-infection is the combination of PbA infection after patent *B. pahangi* 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. Bars with different superscripts indicate significant difference at $P < 0.05$.

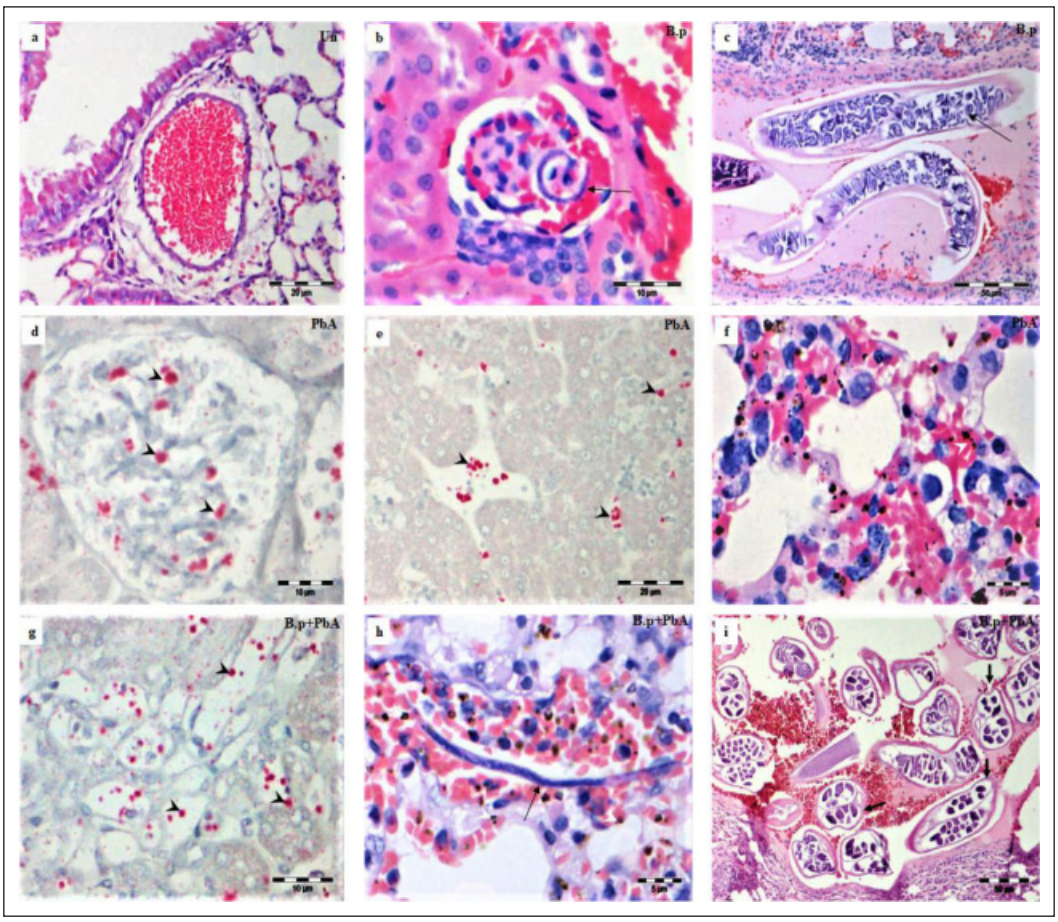

Figure 3. Confirmation of PbA and *B. pahangi* in selected organs. a. lung of an uninfected control gerbil. b. *B. pahangi* microfilaria in kidney of *B. pahangi* infected gerbil (thin arrow). c. cross section of adult *B.pahangi* worm (showing developing microfilaria, thin arrow) in the lung of *B. pahangi* 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 coinfected gerbil (black arrow head). h. *B. pahangi* microfilaria (thin arrow) and malaria pigments (white arrow head) in the lung of coinfected gerbil. i. cross section of adult *B. pahangi* worm (showing internal organs) and malaria pigments in the lung of coinfected 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. B. p: *B. pahangi*, B.p+PbA: coinfected. Bar represents magnification (µm).

7.669, $P < 0.0001$) higher in B.p + PbA-gerbils than PbA-infected gerbils at day 9 pi (Fig. 4b). However, there was no significant difference ($F_{(5, 20)} = 0.0597$, $P > 0.05$) in PbA parasitemia found in the liver of both PbA-infected gerbils and B.p + PbA-gerbils (Fig. 4c).

***B. pahangi* and *P. berghei* ANKA infections resulted in pulmonary edema**

The effect of *B. pahangi* and PbA infections on the lung tissues at different time points were investigated. Lungs were harvested at different time points in all the groups and examined for lesions or injuries. No lesions

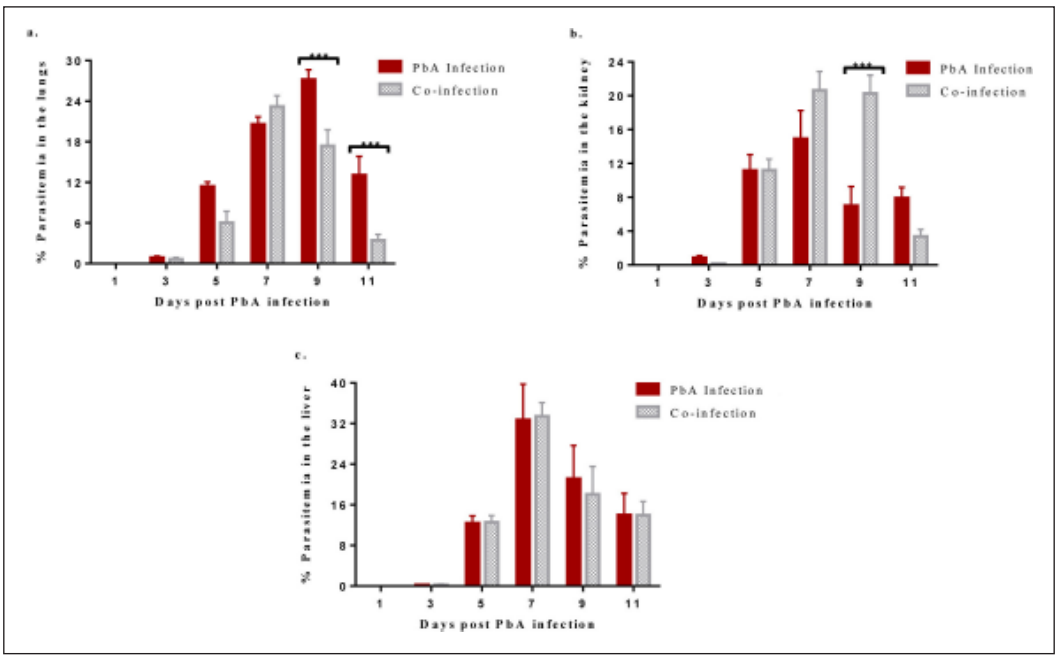

Figure 4. Accumulation of PbA parasitized RBCs (pRBCs) in vessels of selected organs. a. lungs b. kidney and c. liver. At least 100 vessels from each section of the tissue were observed, and pRBCs were counted against uninfected RBCs. Bars represent mean ± S.E.M, N = 5. All data were compared by two-way ANOVA with Sidak's multiple comparison tests for differences between the groups, *** $p < 0.0001$.

Table 1. Pathology observed in the lungs of gerbils

Groups	Time (DPI)	Lesions				
		Increased Alveoli cells	Leukocytes infiltrates	Congestion	Hemorrhagic alveolitis	Hemorrhagic bronchitis
UN	7	0	0	0	0	0
B.p	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
B.p + 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; B.p: *Brugia pahangi* infected gerbils; PbA: *Plasmodium berghei* ANKA infected gerbils; B.p + PbA: gerbils co-infected with *Brugia pahangi* and *Plasmodium berghei* ANKA.

other than infiltrations of leucocytes in the lungs of *B. pahangi* infected gerbils were observed (Table 1). However, lesion such as increase in alveoli cells, leucocytes infiltrations and congestion of cells are commonly observed in both PbA infected and coinfected gerbils (Table 1). In addition, intrapulmonary hemorrhage congestion

was common to PbA infections (Figs. 5d and g), while filarial granulomas were common to *B. pahangi* infections (Figs. 5b and i). The bronchi were highly infiltrated and damaged in all the infections (figs. 5c, f and i). Hemozoin and PbA were found in the alveoli (Fig. 5e) while *B. pahangi* microfilaria were trapped in the alveoli as well (Fig. 5h).

Glomerulonephritis associated with PbA infections

The histopathological assessment of the kidney at different time points revealed no histological changes in the kidney of *B. pahangi* infected gerbils (Table 2). However, PbA infections caused hemorrhage by day 7 pi and increased in mesangial cells, leucocytes infiltration and damaged

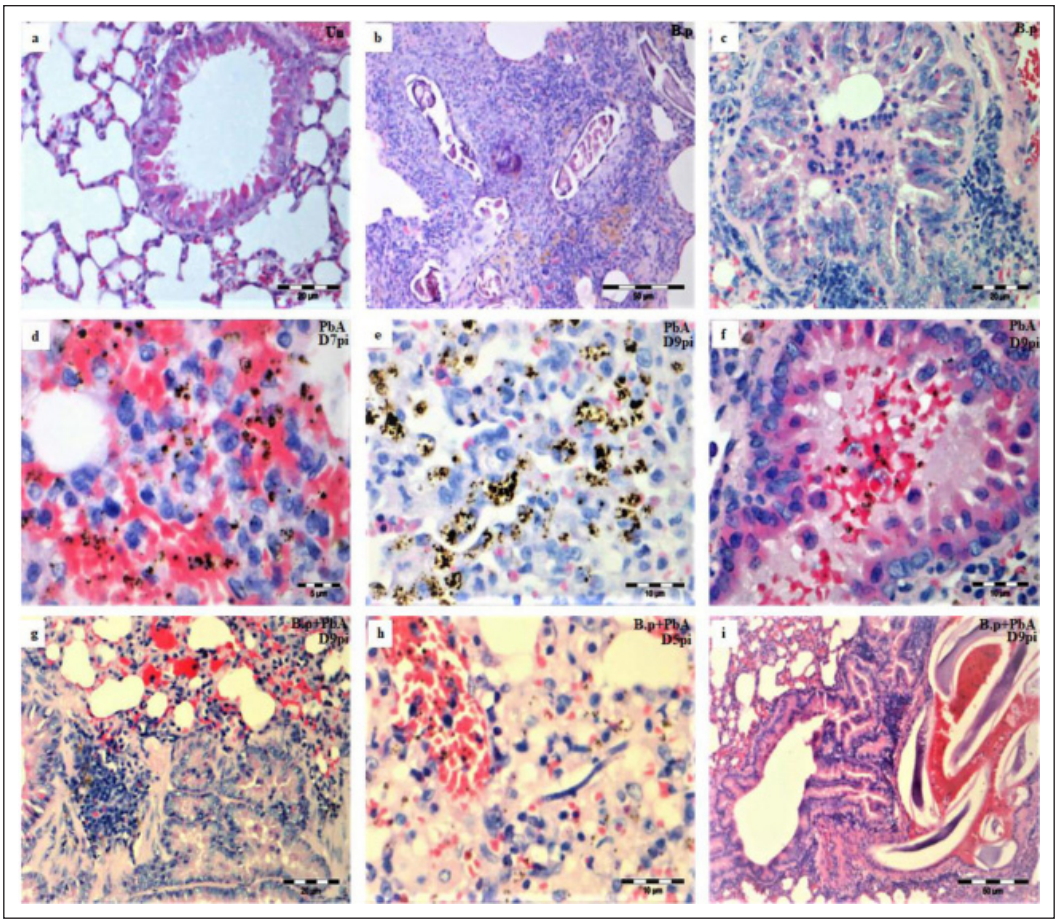

Figure 5. Lung injuries during the course of *B. pahangi* and *P. berghei* ANKA infections in gerbils. a. normal lung in uninfected control gerbils. b. fibrosis and inflammatory reactions from disintegrated worms in *B. pahangi* infected gerbil c. inflammatory cells around the bronchus, together with leucocytes infiltrations in *B. pahangi* infected gerbil. d. hemorrhage and leucocytes infiltrations in PbA-infected gerbils. e. increased cellular activities and intraalveolar macrophages with malaria pigments in PbA-infected gerbil. f. distorted bronchus with *P. berghei* ANKA and pigments in PbA-infected gerbil. g. hemorrhage with increased cellular activities in coinfected gerbil. h. *B. pahangi* microfilaria and *P. berghei* ANKA entrapped in the alveoli of coinfected gerbil. i. filarial granuloma surrounding dead worms with inflammatory reactions in coinfected gerbil. All sections were stained with H and E. B. p: *B. pahangi*, B.p+PbA: coinfected. Bar represents magnification (µm).

Table 2. Pathology observed in the kidney of gerbils

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

The 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; B.p: *Brugia pahangi* infected gerbils; PbA: *Plasmodium berghei* ANKA infected gerbils; B.p + PbA: gerbils co-infected by *Brugia pahangi* and *Plasmodium berghei* ANKA.

glomeruli were all observed in both PbA-infected and B.p+PbA coinfected gerbils by day 11 pi (Fig. 6).

B. pahangi and *P. berghei* ANKA infections induce extramedullary hematopoiesis

Noticeable histological changes during the course of *B. pahangi* and PbA infections were accelerated hematopoiesis in the liver and spleen (Fig. 7). In PbA infections, hemozoin deposits were observed in the Kupffer cells (Figs. 7e and g), while there was congestion of the red pulp in the spleen with hemozoin (Figs. 7f and h).

DISCUSSION

The present study examined the histopathology of *B. pahangi*, PbA and co-infection of *B. pahangi* and PbA on gerbil (*Meriones unguiculatus*) hosts. Results from this study showed that pathologies associated with coinfected gerbils were induced by both *B. pahangi* and PbA infections. Malariail pathology has been ascribed to be the consequence of malaria parasites' ability to bind endothelial cell lining of the brain, lungs, liver and other organs, causing blockage of microvasculature which may results in complicated or severe malaria (Lovegrove *et al.*, 2008; Mishra & Newton, 2009; Rogerson *et al.*, 2007). On the other hand, filarial infection mostly affects the lymphatic system, and 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.

The morphology of liver, spleen and lungs in gerbils showed pigmentation and increased in size in both PbA infected gerbils and coinfected gerbils. These significant increase and pigmentation in organs were not observed in *B. pahangi* infected gerbils, suggesting it to be due to the PbA infection. Previously, significant increases in spleen index have been reported in 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 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 mono

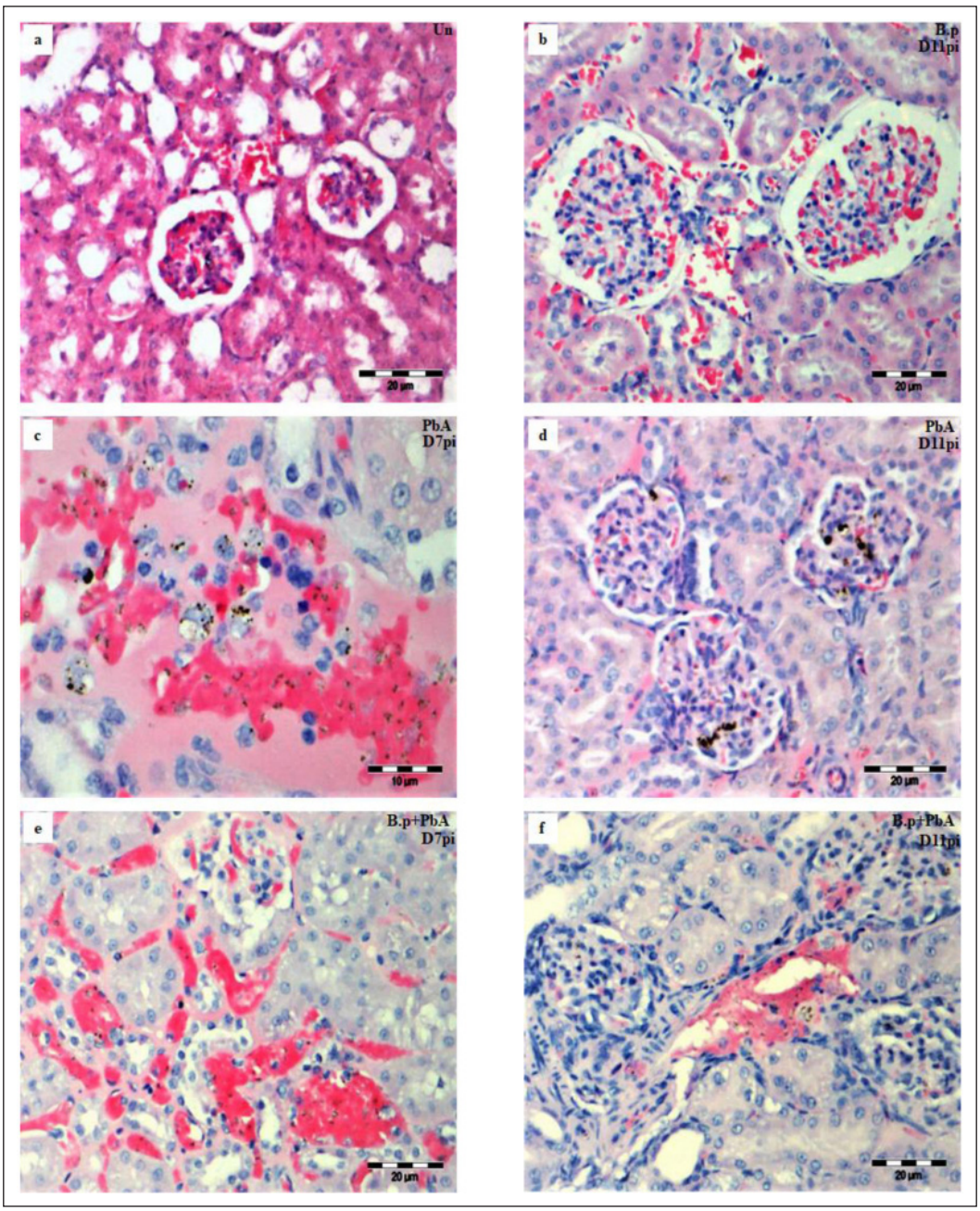

Figure 6. Histological changes in the kidney of *B. pahangi* and *P. berghei* ANKA infections in gerbils. a. Normal kidney architecture from uninfected control gerbils. b. No histological changes in the kidney of *B. pahangi* infected gerbils. c. hemorrhage and infiltrated monocytes in the kidney of PbA-infected gerbils. d. glomerulus with increased mesangial cells and obliterated capillaries in PbA-infected gerbils e. hemorrhage in the kidney of coinfected gerbils. d. distorted and infiltrated glomerulus with increased mesangial cells in coinfected gerbils. B. p: *B. pahangi*, B.p+PbA: coinfected. Bar represents magnification (µm).

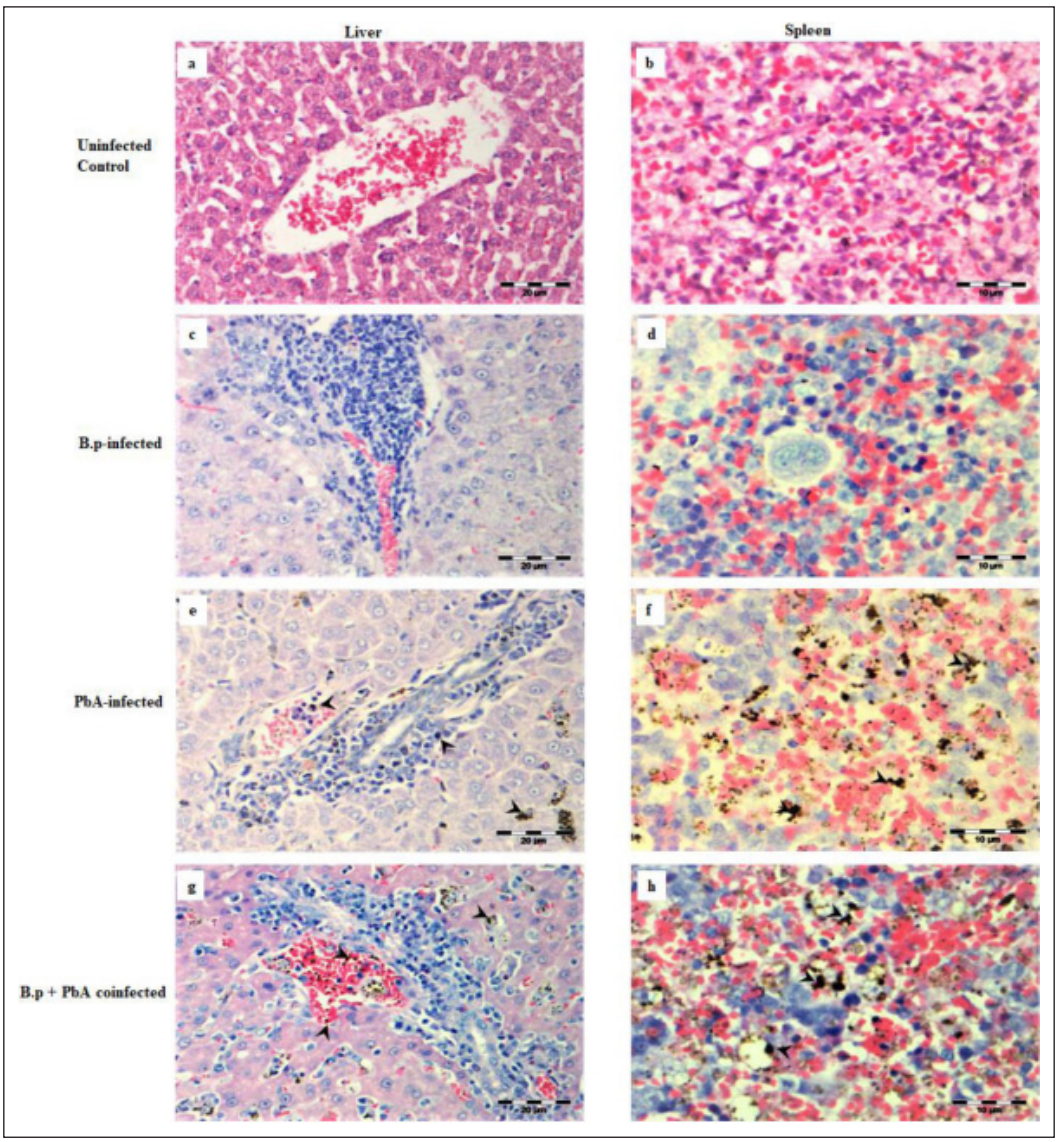

Figure 7. Histological changes in the liver and spleen of *B. pahangi* and *P. berghei* ANKA infections in gerbils. a. normal liver cells structure from uninfected control gerbils. b. normal spleen cells structure from uninfected control gerbils. c. extramedullary hematopoiesis in the liver of *B. pahangi* infected gerbils. d. fibrosis surrounded by inflammatory cells in the red pulp of the spleen in *B. pahangi* 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 coinfected gerbils. h. congestion of red pulp with heavy hemozoin deposits in the spleen of coinfected gerbils. Arrow head show PbA pigments. Bar represents magnification (µm).

infection of *P. yoelii* and *P. chabaudi* (Karadjian *et al.*, 2014). However, the higher splenomegaly reported in these studies differ with the present study, which showed higher splenomegaly in PbA infected gerbils than *B. pahangi*-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* alone (Sangweme *et al.*, 2009).

The spleen serves a key role during malaria infection, where it helps in removal of damaged and pRBCs, stimulates the immune response and aids in production of new RBCs (Engwerda *et al.*, 2005). The removal of both damaged and pRBCs 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 contribute to increase in the organ's weight. Splenomegaly and hepatomegaly increased steadily during malaria infections in gerbils. This has been suggested to be a result of activities of macrophages and dendritic cells in these tissues, 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 pRBCs to sequester in organs, hinder the spleen in removing pRBCs from circulation and thus, aid parasites survival and maintenance (Buffet *et al.*, 2011; Fonager *et al.*, 2012). The present study showed that patent *B. pahangi* infection could not stop accumulation of PbA pRBCs in selected organs, although more parasites were encountered in PbA infected gerbils than *B. pahangi*-PbA coinfected gerbils. However, it has been proposed that the accumulation of malarial parasites in the microvessels of organs will mechanically block the blood vessels (Miller *et al.*, 2002) and the ability of the parasite to cause anemia (Schofield, 2007), this may lead to shortage of blood supplies and invariably organ failure. In the present study, the parasitemia increased steadily before starting to drop from day 7 post PbA infection, which coincided with the peak period of anemia (data to be published) in gerbils.

During the course of filarial and malaria infections lung tissues were damaged. During malaria infection, the binding of pRBCs 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. The developing filarial infective larvae (L3) undergo intralymphatic migration from subcutaneous of the host body through the lymphatic vessels 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 adult worms 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 demonstrated that both adult worm and microfilaria (mf) of *B. pahangi* dwell in the lungs of gerbils. The underlying pulmonary granulomas associated with *B. pahangi* infection could not be reversed or ameliorated in the presence of malaria co-infection. The damage or injury to the lungs in coinfected gerbils appeared to be more severe, partly due to the existing leucocyte infiltrations and granulomatous inflammations afflicted by the filarial infection and combined with the hemorrhagic alveolitis or bronchi due to malarial infection. However, the observations here is similar the report of Karadjian *et al.* (2014), who reported leucocyte infiltration in the lungs of *L. sigmodontis*-infected mice only but affirmed that the filarial worm plays no protective role in the mild lung

injury observed in coinfected mice. Hence, the present study showed that *B. pahangi* plays no protective role on the lesions observed in the lungs of coinfected gerbils and that, both PbA infected and *B. pahangi*-PbA coinfected gerbils suffered severe injury to the lungs during the course of PbA infections.

The histopathology of malaria and filarial infection in humans showed renal failure (Dreyer *et al.*, 1999; 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 not necessarily associated with cytoadherence of the parasite, unlike cerebral malaria. A report on an animal study shows PbA infection did not incur 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). The author's previous report has shown that there is no evidence of cytoadherence of PbA in any of selected gerbil's tissue or organs (Junaid *et al.*, 2017), nevertheless, the possibilities of renal damage or failure cannot be excluded. However, the present study showed the presence of both *B. pahangi* and PbA in the microvessels and glomeruli of gerbil's kidney.

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 *Wuchereria bancrofti* infection (Dreyer *et al.*, 1999). Despite the pathophysiology of the renal disease during filarial infection yet to be known, it has been suggested that the circulating mf might be responsible for mechanical damage done to the glomeruli (which results in hematuria), while inflammatory response has been identified as a potential cause as well (Dreyer *et al.*, 1999). 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 were not determined in the present study and thus, this call for further studies to identify more diagnostic markers associated with pathology of the kidney during co-infection of *B. pahangi* and PbA in gerbils. In Karadjian *et al.* (2014), *L. sigmodontis* showed a protective effect on lesions in the Balb/c kidney, when coinfected with either *P. yoelii* or *P. chabaudi*, however, this was not observed in our study.

CONCLUSION

Histopathological changes in multiple organs of *B. pahangi* and PbA coinfected gerbils were mostly induced by PbA infections except for the acute lung injury in which both parasites synergistically contributed to the severe damage. Notably, the hepatosplenomegaly and leucocytes infiltrations were observed among PbA infected and coinfected gerbil. Filarial granuloma and hemorrhagic alveolitis were also prominent in the lungs of coinfected gerbils. The inflammations and damage to the glomeruli of gerbil's kidney and increased hemapoietic activities of the spleen and liver associated with PbA infections, are of great importance.

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Authors' contributions
OQJ, RM, LTK, and IV conceived and designed the research study, OQJ, KTW and LTK, performed experiments, analyzed and

discussed data, and wrote the paper. RM and IV, reviewed and discussed experimental data, provided materials and wrote the paper. All authors read and approved the final manuscript.

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