Response of outbred albino mice to concomitant *Heligmosomoides bakeri*, *Plasmodium berghei* and *Trypanosoma brucei* infections

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Abstract To investigate the response of mice to concomitant infections with *Trypanosoma brucei* (Tb), *Plasmodium berghei* (Pb) and *Heligmosomoides bakeri* (Hb) infections. Each group of 6 mice was either infected with Pb + Tb + Hb, Pb + Tb, Pb + Hb, Tb + Hb, Pb, Tb, Hb or remained as uninfected controls. Hb infected mice each received 200 infective larvae (L3) of Hb orally, Tb infected mice each received 2×10^{-6} organisms through the intraperitoneal route while Pb infected mice received 1×10^{-5} parasitized red blood cells through the intraperitoneal route. PCV, body weights (BW), faecal egg counts (FEC), Tb parasitaemia, Pb parasitaemia, clinical signs and worm burdens (WB) were determined. FEC were highest in Pb + Tb + Hb and least in Hb group and the difference was significant ($P < 0.05$). WB was significantly higher in mice with concurrent infections. PCV of infected mice was lower than that of uninfected controls and the difference was significant between Pb + Tb + Hb infected and uninfected controls. The difference in weight loss was significant between Pb + Tb + Hb infected and controls. Mortalities occurred in Pb + Tb + Hb, Tb + Hb and Pb + Hb infected mice. Mortalities and low PCV and low BW were indications that concomitant infections were more pathogenic to the mice than single infections, pathogenicity increasing with increasing number of parasite species involved.

Keywords Assess · Response · Outbred albino mice · Concomitant · *Heligmosomoides bakeri* · *Plasmodium berghei* · *Trypanosoma brucei*

Introduction

An understanding of host-parasite interactions is a necessary step towards proper disease control but the task is daunting and even more so in concomitant infections. Unfortunately, the environment of the hosts is often jam-packed with a multitude of parasites and other pathogens and the hosts can consequently be infected with a variety of such parasites (Petney and Andrews 1998; Cox 2001). Unlike in most single infections where the response of the host to the infection can be easily predicted, the situation can be complicated in dual infections and even more complicated in infections involving three or more different parasite species with resultant pronounced effects (Ezenwa et al. 2010; Telfer et al. 2010).

Infecting parasites can either enhance or antagonise the activities of co-infecting parasites thereby altering the course of the infection. *Trypanosoma* species are reported to suppress immune responses of their hosts to co-infecting parasites and to vaccines (Darji et al. 1992; Fakae et al. 1994; Onah and Wakelin 2000; Chiejina et al. 2003). However, a potentiation effect is also known to occur in a conjoint infection of *T. brucei rhodesiense* and *P. berghei yoelii* in mice as the parasitaemia of both parasites increased presumably due to an immune-suppression caused by both parasites in their host (Dallas 1976). *Plasmodium* causes immune suppression in antibody formation and in cell-mediated-immune responses (Kabara et al. 1990). Animals with malaria have thus been shown to have increase susceptibility to *Leishmania mexicana*

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(Coleman et al. 1988), *T. cruzi* (Kabara et al. 1990) and *Toxoplasma gondii* experimentally infected (Kabara et al. 1990).

Immuno-modulation is not a feature limited to protozoa because extensive evidence of immuno-suppression by helminths exists. For instance, C57BL/6 mice infected with *P. yoelii* 17XNL (Py) had a transient and self-limiting malaria infection, however, when the protozoa inoculations were preceded with the *Heligmosomoides polygyrus* (a nematode) the malaria infection was exacerbated resulting in higher parasite burdens and poor survival of the mice due to immune suppression of the mice by the nematode (Tetsutani et al. 2009). *Ascaris* species are widely reported to protect hosts from malaria and its manifestations while hookworms seem to increase the incidence of malaria (Tetsutani et al. 2009). However, in concurrent *Ascaridia galli* and *Pasteurella multocida* or *A. galli* and *Esheriachia coli* infections in chickens, the pathology and clinical signs due to the infections were more severe in the concurrently infected chickens than in those infected with one of the pathogens only (Dahl et al. 2002; Permin et al. 2006). In these two examples *A. galli* was reported to suppress the immunity of the chickens as the pathology and clinical signs became more severe where the nematode preceded the bacteria than when they are given at the same time or *A. galli* after the bacteria (Dahl et al. 2002; Permin et al. 2006).

T. spiralis inoculation at the same time or some days earlier than *T. musculi* infection resulted in an increase in blood parasitaemia in C3H mice. Maximum increases in blood parasitaemia were observed when *T. spiralis* infections preceded *T. musculi* by 5–10 days. Dual infections increased maximum parasitaemia by two- to four-fold, regardless of the degree of resistance of the mouse strain to either *T. musculi* or *T. spiralis*. On the contrary, infection with *H. polygyrus* did not promote *T. musculi* parasitemia as much although the increase in parasitaemia was also proportionate to the dose of *T. spiralis* (Bell et al. 1984). Helminth infections can also interfere with the development of the immune response to vaccines against different diseases (Chruz-Chan et al. 2010).

Our a priori hypothesis was that the pathogenic effects of the infections in the mice would be more severe in the mice with concomitant infections than in the mice with single infections, and that the extent of severity would be more in the mice with three different parasite species. The current study was therefore designed to investigate the response of mice to concomitant *T. brucei*, *P. berghei* and *H. bakeri* infections based on the parasitological (FEC and WB), pathological (PCV and BW) and clinical signs (morbidity and mortality). The hypothesis was made with the hope that if satisfied the results could be applied in designing programmes for control of parasitic diseases of

livestock and man in which case serious measures will be taken in preventing multiple infections of the hosts and proper interpretation of laboratory results.

Materials and methods

Experimental animals

Male outbred albino mice (10–12 week-old) were maintained in cages containing wood shavings with a maximum of 6 mice per cage. Litter was replaced every 2 days with dry fresh wood shavings and equipments were cleaned. The mice were fed ad libitum, with standard pelleted growers mash (Guinea Feed®- compounded by Bendel Feed and Flour Mill-Nigeria), and tap. All mice were screened and treated against gastrointestinal parasites as needed.

Ethical consideration

The ethical conditions governing the use and conduct of experiments with life animals were strictly observed as stipulated by Ward and Elsea (1997), and the experimental protocol was approved by Michael Okpara University of Agriculture, Umudike Animal Ethics Committee.

Body weights of mice

Mice were weighed on day zero (D0) and subsequently every 4 days using a top loading balance (Sartorius GMBH, Gottingen, Germany).

Packed cell volume

Packed cell volume measurements were carried out every 4 days beginning from day zero till the end of each experiment. Mice were bled from the tail directly into heparinized capillary tubes (Camlab Ltd, Cambridge).

Trypanosoma brucei

Trypanosoma brucei used in this study was initially obtained from a clinical case of canine trypanosomosis in the Veterinary Teaching Hospital, University of Nigeria, Nsukka (UNN) and maintained in mice by regular passages until used.

Heligmosomoides bakeri

The gastrointestinal (GI) nematode parasite, *H. bakeri* (formerly *H. polygyrus*) was obtained from the Department

of Veterinary Parasitology and Entomology, UNN where it is maintained in outbred albino mice.

Faecal collection

Faeces for faecal analysis were collected from individual mice by placing them in large faecal collection cups. 1–2 min was enough time for each well-fed mouse to pass out enough faecal pellets for faecal analysis and culture.

Faecal culture

The cultures were set according to the method of Fakae et al. (1994). Pooled faeces were broken up in distilled water in a coffee strainer. The supernatant was siphoned out while the pasty sediment was smeared on moistened filter papers placed in plain petri dishes. Cultures were left for 6–7 days in a refrigerator (4 °C) to ensure optimal hatching especially during the cold dry season (harmatan) or for 7 days at room temperature (20–25 °C) as described by Murua (1975) to ensure optimal hatching of the *H. bakeri* eggs.

Harvesting and preservation of infective larvae (L3)

Cultures were flooded with distilled water at the end of the culture period and left for 1 h and larvae seen at the edges of the filter paper at the bottom of the dishes under the stereomicroscope were rinsed off and placed in Teflon tubes. The larval suspensions were then allowed to settle and excess water decanted. Larval suspensions were stored at 4 °C in shallow water held in tissue culture flasks and used within 4 weeks of recovery.

Preparation of infective doses of L3

The numbers of L3 in ten 50 µL aliquots of well-mixed larval suspensions were counted under a light microscope and the larval concentration determined using the average count. The larval suspension was adjusted with distilled water, as appropriate, to obtain the required concentration in 200 µL.

Infection with *H. bakeri*

The mice were infected orally with *H. bakeri* infective larvae (L3), with 200 L3 suspended in 200 µL of distilled water as shown in the experimental design (Table 1). Properly mixed known volumes of larval suspension were administered to properly restrained mice using automatic micropipette (Finnpipette®; LabSystems Oy, Helsinki, Finland) adapted to take a blunt and slightly curved 19-gauge needle (Fakae 2001).

Infection with *T. brucei*

A donor mouse with high parasitaemia of log₁₀ 8.1 was anaesthetized with diethyl ether (May and Baker, Ltd., England). Approximately 1 mL of blood was quickly taken from the heart of the mouse and put into a bijoux bottle containing 500 µL of ice-cold normal saline, which contained 2 µL of heparin. The parasitaemia of the diluted heart blood was assessed (Mbaya et al. 2009) and adjusted to the working dose of 2×10^{-6} organisms in 0.2 mL of blood (Table 1). Each of the *T. brucei*-infected mice received this dose by intraperitoneal inoculation on D0.

Infection with *P. berghei*

Mice were infected with *P. berghei* by intraperitoneal inoculation with 10⁵ parasitized red blood cells obtained from donor mice (Table 1). 1 mL of blood was taken from donor outbred albino mice and diluted with 5 mL phosphate buffered saline such that that 0.1 mL contained an inoculum of 1×10^{-5} infected red blood cells (Momoh and Longe 2014).

Faecal egg counts

Faecal egg counts were carried out every 2 days using both salt floatation and McMaster techniques during early counts and when counts began to rise respectively (MAFF 1977).

Post mortem worm counts

Post mortem worm counts procedures were conducted according to Ngongeh (2013). Briefly, each mouse was sacrificed with diethyl-ether and the gastrointestinal tract was removed. Three quarters or entire length of the small intestine starting from the duodenum was opened by cutting along its longitudinal axis with a pair of fine scissors. The adult worms from the intestine were recovered by suspending each intestine on a fine thread and dipping into Hanks balanced salt solution (HBSS) in a universal bottle, and the thread supported by fastening its free ends between the universal bottle and its cap, and then incubated at 37 °C. This arrangement allowed the worms to drop freely to the bottom of the bottle after leaving the intestine. Within 2–3 h all worms would have migrated into the saline. The intestine was then discarded, after little agitation to ensure that no worm was being trapped by any two apposed sections of the gut, and fresh saline added. After removal of the intestine the incubation of the worm suspension continued overnight (20 h) to ensure complete disentanglement most of the copulating worms. At the end of the overnight incubation saturated sodium chloride was

Table 1 Experimental design: the grouping and doses of *H. bakeri*, *P. berghei* and *T. brucei* administered to the various groups of outbred albino mice

Group	Age of mice (weeks)	Sex of mice	Dose level of Hb L3	Dose level of Pb infected red cells	Dose level of Tb organisms	Day of autopsy
Hb + Pb + Tb	8–12	Male	200	10^{-5}	2×10^{-6}	20
Hb + Pb	8–12	Male	200	10^{-5}	0	20
Pb + Tb	8–12	Male	0	10^{-5}	2×10^{-6}	20
Hb + Tb	8–12	Male	200	0	2×10^{-6}	20
Hb	8–12	Male	200	0	0	20
Pb	8–12	Male	0	10^{-5}	0	20
Tb	8–12	Male	0	0	2×10^{-6}	20
Uninfected	0	Male	0	0	0	20

Hb + Pb + Tb, infected with *H. bakeri*, *P. berghei* and *T. brucei*; Hb + Pb, infected with *H. bakeri* and *P. berghei*; Pb + Tb, infected with *H. bakeri* and *T. brucei*; Hb + Tb, infected with *H. bakeri* and *T. brucei*; Hb, infected with *H. bakeri*; Pb, infected with *P. berghei*; Tb, infected with *T. brucei*; L3, infective larvae of *H. bakeri*

added to the Hanks saline containing the worms to make up a 30% v/v solution. The worms then died within 30 min of this treatment, relaxing the tight spiral coils characteristic of the live worms, thus making counting easier. The volume of the suspension was reduced to 5 mL and poured into a clean ruled Petri dish for easy counting of the worms.

Trypanosoma parasitaemia

Mice were bled from the tail for the estimation of parasitaemia (Fakae 2001). At higher levels of infection, microscopic fields (400× magnification) of wet blood film were matched against standard charts, but where fewer organisms were present, the number of *Trypanosoma* in 5, 10, or 20 such microscopic fields were counted and the equivalent number in one millilitre of blood was read from a standard table (Fakae 2001). The number of organisms per field was estimated and expressed as $\log_{10}$ per millilitre of blood.

Plasmodium parasitaemia

Parasitaemia was monitored by examining Giemsa stained blood smears every 4 days beginning from day four post infection. Thin blood smears were prepared on microscopic slides with blood from the tail. The slides were air dried, fixed with methanol and stained with Giemsa stain. The parasitaemia was determined by counting a minimum of three fields per slide with 100 RBC per field (Zucker and Campbell 1993). Parasitaemia = number of parasitized RBC/Total number of RBC examined.

Statistical analysis

Statistical analysis was carried out using SPSS version 12.0.1. Data were analysed using analysis of variance

(ANOVA) in general linear model (GLIM) and results were summarised as means with standard errors of means (SEM) and *P*-values less than 0.05 were taken as significant.

Results

Faecal egg counts

The Hb infection was patent in all the mice infected with Hb on D10. FEC generally rose from D10 to D12 before fluctuating to the end of the study on D18. The rise in FEC in the Pb + Tb + Hb mice was steep reaching a high peak on D12 before fluctuating to D18. FEC was highest in the Pb + Tb + Hb and least in the Hb group and the difference was significantly higher ($P < 0.05$) on D12 while it was moderate in the Pb + Hb and Tb + Hb groups in the acute phase (Fig. 1). There was however no significant difference ($P > 0.05$) in FEC between groups.

Worm burden

The WB varied across various groups being least in the Hb, moderate in the Pb + Hb and Tb + Hb and highest in the Pb + Tb + Hb. The WB of each group was significantly different ($P < 0.05$) from that of other groups (Fig. 2).

Packed cell volume

There was a general tendency for the PCV to drop in all groups, however, apart from the Pb-only infected group all other infected groups suffered severe drops in PCV on D12 (Fig. 3). The greatest drop in PCV was observed in the Pb + Tb + Hb group and the difference was significant

Fig. 1 Mean changes in FEC of mice infected with Pb + Tb + Hb, Pb + Hb, Tb + Hb and Hb only

Fig. 2 Mean worm burdens of mice infected with Pb + Tb + Hb, Pb + Hb, Tb + Hb and Hb

($P < 0.05$) compared to the uninfected control on D12 (Fig. 3a). The Pb-only group had the least drop in PCV (Fig. 3b) on D12. The PCV of the Pb + Tb + Hb was also significantly lower ($P < 0.05$) than that of the Pb + Tb, Pb + Hb on D16 and D20 (Fig. 3a).

Body weights

Body weights generally fluctuated with major falls in body weights on days 10, 15 and 20 in the Pb + Tb, Pb + Hb and Pb + Tb + Hb infected groups (Fig. 4a). The loss in weight was more severe in the concurrent infected animals and the loss continued to D20 in the group with triple infections while it fluctuated in the dual infected groups (Fig. 4a, b). The weight loss in the Pb + Hb was significant ($P < 0.05$) on D15 when compared to the other groups.

T. brucei parasitaemia

T. brucei infections became patent in all mice infected with the parasite but while parasitaemia remained fairly low and steady in other groups it fluctuated markedly in the

Fig. 3 a Mean changes in PCV of mice infected with Pb + Tb + Hb, Pb + Tb, Pb + Hb, Pb and uninfected controls. **b** Mean changes in PCV of mice infected with Tb + Hb, Tb, Hb, Pb and uninfected controls

Pb + Tb + Hb group attaining great peaks on D12 and on D20 when the experiment ended (Fig. 5).

Plasmodium berghei parasitaemia

The *P. berghei* infection was patent on D4. Parasitaemia fluctuated but generally rose from D4 to D16 (Fig. 6). The parasitaemia was significantly higher ($P < 0.05$) in the Pb + Tb + Hb and Pb + Hb than in Tb + Pb and Pb on D8.

Mortalities

Mortalities occurred in some groups of infected mice before the end of the study on D20. They were one, two and three mortalities in the Pb + Hb, Tb + Hb and Pb + Tb + Hb respectively while there was no mortality in the other groups. This was summarised as percentage mortality (Fig. 7).

Discussion

The results suggest that the infections were more pathogenic to the mice with multiple infections. Concurrent infections generally enhanced the worm burdens since the

Fig. 4 a Mean changes in body weights of mice infected Pb + Tb + Hb, Pb + Tb, Pb + Hb, Pb and uninfected controls. b a: Mean changes in body weights of mice infected with Tb + Hb, Tb, Hb and uninfected controls

Fig. 5 Mean changes in *T. brucei* parasitaemia of mice infected with Pb + Tb + Hb, Pb + Tb, Tb + Hb and Tb

Hb-only infected group harboured significantly less worms than any other group. The intensity of the nematode infection seemed to be dependent on the number of infecting parasite species as the WB was generally high in the groups of mice with dual and triple infections and low in the Hb-only infected group of mice. There was thus a sort of summation effect of the pathogenicity of the parasites in the mice particularly as morbidity and mortality were more common in the animals with concomitant infections. It was particularly obvious as mice infected with

Fig. 6 *P. berghei* parasitaemia of mice infected with Pb + Tb + Hb, Tb + Pb, Pb + Hb and Pb

Fig. 7 Percentage mortality of mice infected with Pb + Tb + Hb, Pb + Tb, Pb + Hb, Tb + Hb, Pb, Tb, Hb and uninfected controls

all the three parasite species suffered the highest rate of mortality (50%). This finding is in line with the view that multiple species infections may increase susceptibility to other parasites (Druilhe et al. 2005; Mwangi et al. 2006; Nacher 2004).

The influence of the number of parasite species involved was also reflected in the PCV in which the mice infected with 3 different parasite species had the lowest PCV. Lewinsohn (1975) had also reported severe anaemia in mice with *P. berghei yoelii* and *Schistosoma mansoni* mixed infections although in her study the anaemia in the *P. berghei yoelii*-only infected mice was also severe unlike in the current study where the anaemia due to *P. berghei* alone was mild compared to that due to Tb. Some combinations of the mixed infections were more pathogenic than others as clearly seen in the mice infected with all the three parasites. Some dual combinations especially the Pb + Hb and Tb + Hb combinations were also highly pathogenic given that they caused one and two mortalities of the mice respectively unlike in the Pb + Tb infected mice where no mortality occurred. On the other hand no

mortality occurred in the singly-infected mice and this is suggestive of the relative ability of the mice to tolerate the parasites for some time when they occur singly.

Mixed infections have also been shown to enhance the pathogenicity of parasites in humans resulting in high parasite intensities in individuals harbouring more parasite species (Booth et al. 1998; Brooker et al. 2000). Ezeamama et al. (2012) have reported that children with low intensity multiple infections involving *Schistosoma japonicum*, *Trichuris trichiura*, *Ascaris lumbricoides* and *Necator americanus* were more prone to anaemia than those with single-species infections.

The higher WB in concurrently infected groups of mice in comparison with the Hb-only infected mice is an indication that the concurrently infecting parasites with Hb potentiated its infectivity. This potentiation might have been as a result of immune-downregulation of the mice by the Pb and Tb allowing high rate of survival of the worm (Hb) in the mice. The higher WB and FEC recorded in the current study in concurrent Hb and Tb infected mice compared to their Hb-only infected counterparts is similar to the findings of Ngongeh (2013) who reported that mice with Tb and Hb concurrent infections had higher FEC and WB than was found in the Hb-only infected mice and this was postulated to be due to immunosuppression of the mice by the trypanosome thereby enhancing the nematode establishment and fecundity.

Mice with *Trichuris muris* and *T. brucei* co-infections were also reported to be unable to expel the nematode which it usually expels in single infections due to immunosuppression by the protozoan (Philips and Wakelin 1974). Similar findings have been recorded in WAD goats where goats infected with both *T. congolense* and *Haemonchus contortus* had depressed antibody response to *H. contortus* and increased strongyle FEC (Faye et al. 2002). Chiejina et al. (2005) had also shown that WAD goats concurrently infected with *H. contortus* and *T. brucei* had higher WB and increased FEC. A potentiation effect was also reported in concurrent infections of *T. rhodensiense* and *Plasmodium berghei yoelli* in mice and the effect was attributed to possible immune depression by both parasites in their host (Dallas 1976). *S. mansoni* had enhanced *P. berghei* infection and lead to increased parasitaemia and mortality (Mengistu et al. 2004). It is therefore possible that the exacerbated effects of the infections in most of the mice with concurrent infections demonstrated by both parasitological (FEC, WB and parasitaemia) and pathological (PCV, body weights) parameters were both due to various influences of the infecting parasites such as their immunosuppressive effects on the hosts allowing their co-infecting parasites to establish and flourish, and also due to the direct physical damage caused by the parasites such as blood sucking (due to Hb), haemorrhages (due to Hb) and

lysis of red blood cells (due to Tb and Pb) with a resultant heavy toll of infection on the hosts.

The falling PCV observed in all mice including the uninfected control cannot be readily explained. However, infected mice showed a conspicuous fall in PCV when compared with their uninfected counterparts. The extent of decline of PCV was however dependent on the type of infecting parasite or parasite combinations involved in the various groups as earlier mentioned. The PCV drop was notably most severe in the mice infected with all the three parasite species while it was moderate in the dual infected groups. Tb seemed to be the most anaemia-causing parasite of the three parasites involved since it induced a drop in PCV almost similar to that induced in mice with three parasite species. All the three parasites studied here are anaemia-inducing either by blood sucking and haemorrhage (Hb) or by haemolysis (Tb and Pb) and this was probably why the PCV decline was most severe in the group of mice with triple infections. A sort of summation effect might have occurred in the process of inducing anaemia.

Considering the mortalities, the low PCV and low BW, it is clear that concurrent infections were more pathogenic to the mice than single infections. Given that the highest number of mortalities occurred in the mice infected with the three different parasites and that mice of the group also suffered the greatest PCV and BW declines is suggestive that the Pb + Tb + Hb was the most pathogenic combination and that pathology correlated with the number of infecting parasite species. There was therefore a sort of an overall build up of the pathogenic effects of the infecting parasites which increased as their number of parasite species increased. Therefore, there is a high risk of more severe pathology to the hosts harbouring concomitant infections. The a priori hypothesis was therefore satisfied since concomitant infected mice particularly those that harboured three parasites suffered the greatest effects of infection shown by pathological (PCV and BW) and parasitological (FEC and WB) parameters and clinical signs (mortality and morbidity).

The various combinations of parasites in the concomitant infected mice might have had either antagonistic or synergistic effects with each other but the overall effect was that the pathology due to the infections was more severe in mice with concomitant infections than in those with single infections. The mortalities, low PCV and BW were an indication that concomitant infections were more pathogenic to the mice, pathogenicity increasing with increasing number of parasite species. There is need therefore to routinely monitor both animal and human hosts for the presence of parasites and treat when present especially when they occur as mixed infections even at low intensities in order to improve their health and

performance. Control efforts aimed at minimizing the number of different parasite species infecting hosts should be implemented particularly where many of the infections are endemic.

The findings here support some earlier findings but further reiterate the significance of multiple infections in man and his domestic animals and the need to diagnose and approach the control of such infections based on their impact on their hosts. This study is particularly special in that it is more close to the kind of cocktail of infections that occur in individual hosts in their open environment in nature and points an accusing finger to parasite control programmes that hastily consider particular parasites in isolation.

Author Contributions LAN Designed and oversee the smooth running of the study. Performed the infections and recorded and carried out quality control of the data. Also, performed faecal egg counts, read *Plasmodium berghei* slides (*P. berghei* parasitaemia), conducted worm counts and preparation and submission of the manuscript. AO Performed *T. brucei* infections, prepared smears for *P. berghei* parasitaemia, taking of *T. brucei* parasitaemia and feed procurement and feeding. MIW Responsible for taking *T. brucei* parasitaemia, body weight readings of mice and faecal egg counts. SKG He ensured the equipment were functional and ready for use all the time. He participated in collection of specimens and taking the PCV and *Plasmodium* parasitaemia of the mice.

Compliance with ethical standards

Conflict of interest We declare that we do not have any conflict of interest.

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