

# Validation of the Infectra<sup>®</sup>-Kit in Malaria Transmission Studies Using *Plasmodium Berghei*

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**Abstract:** Pre-clinical transmission assays are essential for proof-of-concept for transmission blocking strategies but are hazardous to laboratory personnel and animal hosts as it entails exposure of live rodents to infected vectors. Conventional transmission assay methods include the use of anesthesia (associated with undesired side effects). In addition, animal handlers risk being bitten by experimental animals and vectors during anesthesia due to a lack of safe and effective alternatives. Robustness of rodent to vector transmission was determined by comparing the number of oocysts. Vector-to-rodent transmission was determined by measuring parasitemia, gametocytemia, changes in body weight and survival time. A completely randomized design was used in this study. Rodent-to-vector transmission was analyzed by log linear model. Fecundity, gametocytemia, parasitaemia and changes in body weight were analyzed by regression analysis. Survival times were analyzed Kaplan-Meier method for determination of survival distribution function. Rank test of homogeneity were used to determine the effect of restraining method infection on survival times. There was no significant difference ( $p < 0.001$ ) in fecundity of mosquitoes fed on anesthetized mice;  $122 \pm 22.1$  eggs compared to INFECTRA<sup>®</sup>-Kit group with  $110 \pm 14.1$  eggs. Oocyst production increased gradually though not significantly ( $p < 0.001$ ) in both groups of mice with the number of mosquitoes. The INFECTRA<sup>®</sup>-Kit group increased from  $2.7\% \pm 0.3$  (1 mosquito) to  $9.3\% \pm 0.3$  (6 mosquitoes), the conventional group was  $3.7\% \pm 0.3$  to  $8.6\% \pm 0.3$  (6 mosquitoes). Parasitemia progression was characterized by two waves in INFECTRA<sup>®</sup>-Kit and three waves in the conventional group. The highest parasitaemia peak was 22% attained on 22dpi for the INFECTRA<sup>®</sup>-Kit and 17.8% attained on 26 dpi for the conventional group. Gametocytes were detected on 16 dpi in both groups and thereafter increased significantly ( $p < 0.001$ ) with dpi. In the INFECTRA<sup>®</sup>-Kit group, gametocytemia was represented by two oscillations while the conventional group was three cycles with peak gametocytes increasing with each subsequent peak. Disease progression was higher and survival times shorter with INFECTRA<sup>®</sup>-Kit than with anesthetized mice and there was no significant difference ( $p < 0.05$ ) between the two methods in body weight and gametocytemia. INFECTRA<sup>®</sup>-Kit is equivalent to that of anesthesia method but more advantageous given the more ethical and humane treatment of animals.

**Keywords:** Infectra<sup>®</sup>-Kit, Transmission Assays, Humane Treatment of Animals, Anesthesia

## 1. Introduction

Malaria is a mosquito-borne infectious disease of humans and other animals caused by parasitic protozoans (a type of

unicellular microorganism) of the genus *Plasmodium* (Bartoloni *et al.*, 2012). Commonly, the disease is transmitted via a bite from an infected female *Anopheles* mosquito which introduces the organisms from its saliva into the person's circulatory system. In the blood, the organism travels to the

liver to mature and reproduce. Symptoms associated with malaria include fever and headache and in severe cases infection progress to coma and death. The disease is widespread in tropical and subtropical regions in a broad band around the equator, including much of Sub-Saharan Africa, Asia and the Americas (Bartoloni *et al.*, 2012). The World Health Organization estimated that in 2010; there were 219 million documented cases of malaria. That year alone, the disease killed between 660,000 and 1.2 million people (WHO, 2012), many of whom were children in Africa. Malaria is commonly associated with poverty and therefore economically important. Management of malaria is basically through chemotherapy or by prophylaxis mainly to travelers making trips to malaria-endemic countries or regions. Severe malaria is treated with intravenous or intramuscular quinine (Sinclair *et al.*, 2012). Since mid-2000s, the artemisinin derivative artesunate, which is superior to quinine (WHO 2006) has been in use in treatment in both children and adults and is given orally in combination with a second anti-malarial such as mefloquine (WHO 2006). Resistance has developed to several anti-malarial drugs; for example, chloroquine-resistant *P. falciparum* has spread to most malarial areas and emerging resistance to artemisinin has become a problem in some parts of Southeast Asia (Bartoloni *et al.*, 2012; Nadjm *et al.*, 2012). There is no effective vaccine currently existing, although efforts to develop one are ongoing (Birkett AJ *et al.*, 2013). Vaccines that interrupt malaria transmission (VIMT) have been highlighted as an important intervention, including transmission-blocking vaccines that prevent human-to-mosquito transmission by targeting the sexual, sporogonic, or mosquito stages of the parasite (SSM-VIMT) (Nunes *et al.*, 2014). *Plasmodium falciparum*, one of the five *Plasmodium* species that infect humans, is the most lethal parasite and contributes to the majority of deaths from the disease (Greenwood *et al.*, 2008). However, the host specificity of this human malaria parasites represents a major constraint on *in vivo* studies (Ozwar *et al.*, 2003, Vaughan *et al.*, 2012) making studies on drugs and vaccine development using laboratory animals difficult. Murine malaria parasites like *Plasmodium berghei* is therefore used in these type of studies as it is similar to human malaria parasites in most essential aspects of morphology, physiology and life cycle studies aimed at the development of new drugs or a vaccine against malaria (Carter and Diggs, 1977).

Infection transmission studies using animal models are important for demonstrating the critical proof-of-concept for host protection and transmission blocking interventions at the preclinical phase of development. For example, in malaria studies this involves the challenge of healthy animals using infected mosquitoes for demonstrating protection in the animal model in the case of vaccine development and chemotherapeutic prophylaxis whereby these studies equally involve feeding of uninfected mosquitoes on infected animals to demonstrate the efficacy of gametocidal drugs, transmission blocking vaccines or vector targeting interventions that express mosquito refractoriness to

*Plasmodium* infection in the mosquitoes (Blagborough *et al.*, 2013).

Malaria transmission blocking experiments are routinely carried out in the laboratory using laboratory mice which are chemically restrained (immobilized) by anesthesia after which mosquitoes in a cage are fed by placing on the immobilized mouse and in the process, infection is transmitted (Darcy & Photini, 2005). Information on the effect the anaesthetizing chemical may have on the parasite is not adequate. Previous studies have for example shown that fecal glucocorticoid levels increases after anesthesia (Palme *et al.*, 2005). Further studies showed that serum electrolytes, glucose, and insulin are altered after general anesthesia (Tanaka *et al.*, 2009). When animals are chemically immobilized, they may undergo some or all of a series of acute stressors including pursuit, restraint, pain, fear and anxiety, all of which are capable of inducing harmful responses and pathological changes on laboratory animals which are likely to affect the outcomes (Jewell & Alibhai, 2010) of the study. Immobilizing drugs have the potential to disturb normal regulatory systems, particularly respiratory and thermo-regulation, which in turn can lead to negative outcomes such as respiratory depression, over-heating (hyperthermia), lowered blood pH (acidosis) and oxygen deficit (hypoxemia) can lead to neurological or myocardial problems and multi-organ failure and can lead to death (Armeno & Caulkett, 2008). Drugs have a range of effects from those which produce a widespread muscular paralysis while the animal is fully conscious, to those which produce unconsciousness with anesthesia causing a lack of sensation or pain (Armeno & Caulkett, 2008).

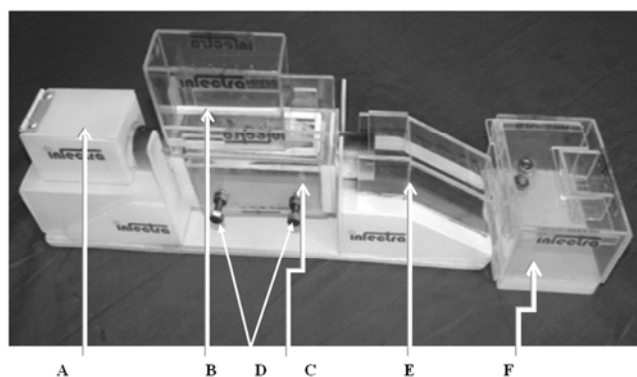
Furthermore, chemical method of immobilizing the rodent is not suitable as it negates the natural method of parasite transmission. Other than chemical immobilization, the mouse can physically be restrained during transmission experiments by holding the rodent with one hand and placing the vector while in a cage on the ventral region of the rodent with the other hand. This method of restraining subjects the personnel to the risk of being bitten by either the rodent or the infected vector. The rodent is equally subjected to stress related death resulting from prolonged holding. Although chemical restraining techniques are sometimes used during transmission studies (Secundino *et al.*, 2012; Caljon *et al.*, 2006), they have limitations since chemicals can induce fly mortality (Kibugu *et al.*, 2010; IAEA, 2000; Feldmann *et al.*, 1994) and alter animal physiology (Wheler *et al.*, 2010) negating host-mosquito interaction. To counter these restraining challenges (chemical and physical), INFECTRA®-Kit a mouse and vector restrainer which allows the two to naturally interact as in the field was developed and its applicability validated using tsetse flies and Swiss White Mice (Ndungu *et al.*, 2013). The applicability of this kit with other vectors, malaria transmission blocking studies has not been tested which forms the objective of this study. It is anticipated that the industrial applicability of this kit in malaria transmission and blocking experiments will provide the scientists researching on vector borne diseases with an

opportunity to conduct infection transmission experiments naturally in the laboratory mimicking the field scenario. The effect the anaesthetizing drug may have on the virulence of the parasite is missing or inadequate and it is further anticipated that results from this study will send some insight on this.

## 2. Materials and Methods

### 2.1. Infectra®-Kit

The INFECTRA®-Kit apparatus (Fig. 1) was received from the Kenya Agricultural and Livestock Research Organization (KALRO) – Biotechnology Research Institute (BRI). The kit operates as outlined by Ndungu *et al.*, 2013.



**Figure 1.** Infection Transmission Kit (INFECTRA®-Kit). Briefly, lid 'A' opens up into a space through which the mouse is introduced, it then moves to the enclosure 'C' where it is restrained/immobilized by fastening the enclosure with screws 'D'. The mosquito vector in cage 'B' is then placed on the immobilized mouse. After vector engorgement is visually confirmed, the mouse is released through ladder 'E' into a resting cage 'F' from where it is picked and transferred back to the mouse cage.

### 2.2. Anesthetization of Rodent –Chemical Method of Restraining

Each mouse was chemically restrained using 6% sodium pentobarbitone intraperitoneally injected at 0.1ml/kg body weight per donor mice

### 2.3. Mosquito Colony

Five hundred, 5-7 day old female *Anopheles gambiaes. s.* mosquitoes (Kisumu strain) were kept in improvised cages fashioned from 2 liters (volume) plastic containers with top netting were maintained at  $27\pm 2^{\circ}\text{C}$  and  $70\pm 10\%$  RH at a 12:12 L:D photoperiod and were blood fed on 6 clean mice daily. Eggs were laid on wet filter paper and transferred to rearing pans for hatching. The hatched larvae were maintained at  $32\pm 2^{\circ}\text{C}$ ,  $90\pm 10\%$  RH, 12:12 L:D photoperiod in rectangular plastic pans of 30 x 40 cm that were flooded with dechlorinated tap water and fed on Tetramin® fish food.

### 2.4. Laboratory Mice

Thirty three (33) Swiss albino mice, aged between 4-6 weeks and weighing between 22-26g were purchased from

the KEMRI Animals Breeding Unit and housed in the KEMRI experimental animal care unit in a standard 67–75 sq. inch cage throughout the experiments. The mice cage facilities were regularly inspected for proper lighting, ventilation, temperature, foot bath sterilization according to KEMRI standard operating procedures. The mice were acclimatized for 1 week before commencement of the experiments. They were maintained on commercial feed (Mice pellets®, Unga Ltd., Kenya), water provided *ad libitum* and wood chippings used as bedding material.

### 2.5. Parasite

*Plasmodium berghei* ANKA - 15% parasitized RBCs stored at  $-80^{\circ}\text{C}$  from cardiac puncture of a previous donor mouse were retrieved, allowed to thaw and intraperitoneally inoculated using a 22G needle with  $1 \times 10^4/\text{ml}$  virulent wild type into 3 donor mice which were then monitored daily for parasitaemia development. The experimental mice, six (6) per restraining method were then intraperitoneally injected from the infected donors having 10% parasitemia. The development of gametocytemia was then monitored daily from the mouse tail until reaching its peak of 10% for successful infection of mosquitoes (malaria transmission).

### 2.6. Fecundity

Mosquitoes were randomly picked  $n=126$  ( $21 \times 6$ ) mosquitoes via an aspirator from the 500 mosquitoes and starved for 24 hours prior to feeding on mice INFECTRA®-Kit and conventionally restrained. For each of the two restraining methods, twenty one (21) non-infected female mosquitoes were divided into six groups of 6, 5, 4, 3, 2 and 1. Six (6) mice were each placed into vector chambers of INFECTRA®-Kit. Each of the six groups of mosquitoes was then placed on each of the restrained mouse and the same was replicated with the conventionally restrained mice. In both restraining methods, mosquitoes were allowed to blood feed for 20 minutes until engorgement was attained. Five days post infection; the laid eggs were photographed and counted using a grid. Triplicate assays were performed per method for purposes of validation.

### 2.7. Mosquitoes Infection –(Rodent to Vector Transmission)

One hundred twenty six (126) 5-7day old, non-infected female mosquitoes were starved for 24 hour prior to feeding on infected mouse. Twenty one (21) mosquitoes ( $21 \times 3$  replicates) for each restraining method and were placed in groups of 6, 5, 4, 3, 2, and 1 into vector chambers. These groups of mosquitoes were individually fed on a single infected mouse with a gametocytemia of 10% while restrained using the INFECTRA®-Kit for a period of 20 minutes. The same was replicated using the conventionally restrained mice. Engorged abdomen was considered indicative of successful feeding as previously reported (Okoth *et al.*, 2006). Feeding was repeated for any mosquito which failed to imbibe and was replaced with another to ensure the appropriate number of infected mosquitoes per

group was achieved. Three replicates of each assay (restraining method) were done for purposes of validation totaling 252 (42 mosquitoes per method). Mosquito midgut and salivary glands were dissected as described by Coleman *et al.*, 2007 but with few modifications whereby only dissecting pins were used *in lieu* of fine tipped forceps. Simple dissecting pins were fashioned from 1ml, 22G insulin needles manufactured by Becton Dickson and Company (BD Micro-Fine™ *plus*, Franklin Lakes NJ, USA). The oocysts were counted in a 4x4 grid whereby the number of oocysts per mm<sup>2</sup> x10<sup>4</sup> (mean range of 5-254) per infected mosquito was recorded.

## 2.8. Mice to Mosquito and to Mice Transmission

Twelve 4-6 week old and weighing 22-24g Swiss Albino mice were tail marked and randomly segregated into three (3) groups of six (6) mice each (n=6) using blind randomization. 136, 5-7day old, non-infected female *Anopheles gambiae* mosquitoes were received from KEMRI insectary unit, starved for 24 hours and then blood fed on the 3 donor mice infected with *P. berghei* at 10% parasitemia. Fourteen (14) days post feeding 10 mosquitoes were blindly aspirated at random and were knocked down by cooling at -20°C. The mosquitoes were then dissected to confirm the presence of sporozoites in the salivary gland. Infected mosquitoes were fed as follows: Group 1 and 2, six mice each restrained using INFECTRA®-Kit and conventionally respectively were each fed on 21 mosquitoes (21x3 replica); group 3, six uninfected and unrestrained control mice were not exposed to the infected mosquitoes. Infected mice were monitored for 30 dpi for parasitaemia and gametocyte development and surviving mice euthanized and disposed as required by the IACUC.

Total parasitaemia (percentage of red blood cells infected) and gametocytemia were monitored daily starting seven (7) days post-infection using thin blood smears of tail blood fixed in methanol and stained in 10% Giemsa. Blood was collected by pricking the tip of the tail using a sharp pair of scissors and thin smears made on a frosted slide and stained in Giemsa as outlined by Malaria Working Party, 1997. Triplicate Giemsa-stained smears were made and examined under the light microscope for the presence gametocytes and *Plasmodium berghei* parasites in the red blood cells. The infected mice were monitored for the prepatent period (first detection) in the peripheral blood parasitaemia progression

and gametocytemia were expressed as a percentage of at least 2000 RBCs.

### 2.8.1. Body Weight

Body weight changes as infection progressed were recorded thrice a week using digital weighing balance.

### 2.8.2. Survival Time

The survival time for each experimental mouse was monitored daily for 30 days post infection when the experiment was terminated. For mice surviving beyond this period, survival time was recorded as 30 days and categorized as censored data.

## 3. Results

### 3.1. Fecundity

Mosquitoes fed on mice restrained using the two methods successfully laid eggs (Fig. 2). The number of eggs laid by mosquitoes fed on INFECTRA®-Kit restrained mice had a mean of 110 ± 14.1 eggs compared to 121±22.1 eggs in the conventional group of restrained mice. Mean fecundity in INFECTRA®-Kit increased from 68±11 (1 mosquito) to 191±25 (6 mosquitoes) while the conventional group increased from 60±5.2 (1 mosquito) to 195±19 (6 mosquitoes) (Fig. 3, Table 1). It was observed that number of eggs was highly significant (p<0.001) depending on the number of mosquitoes with number of eggs significantly increasing with number of mosquitoes. Number of eggs in the INFECTRA®-Kit group was significantly (p<0.05) lower than that in the Conventional group irrespective of the number of mosquitoes:  $\text{Log (Eggs)} = \beta_0 \text{ (Constant/intercept)} + \beta_1 * (\text{INFECTRA}^\circ\text{-Kit}) + \beta_2 * \text{log (Conventional)}$ ,  $\text{Log (Eggs)} = 4.07 + 0.19 + 0.23$



**Figure 2.** Photograph image of mosquito eggs on petri plates where 1i – 1 mosquito INFECTRA-Kit, 6i – six mosquitoes INFECTRA®-Kit, 1c – 1 mosquito Conventional, 6c – six mosquitoes Conventional.

**Table 1.** Number of eggs laid by mosquito fed on mice restrained with the two methods.

Restraining method						
INFECTRA®-Kit	Paper Cup #	A	B	C	Total	Mean
	1	49	86	69	204	68± 11
	2	59	56	77	192	64±6.6
	3	76	130	99	305	102±15.6
	4	98	94	139	331	110 ±15
	5	111	151	117	379	126 ±12
	6	180	155	239	574	191± 25
						110 ± 19.0
Conventional	Paper Cup #	A	B	C	Total	Mean
	1	61	68	50	179	60 ± 5.2

Restraining method						
INFECTRA®-Kit	Paper Cup #	A	B	C	Total	Mean
	2	67	178	63	308	103 ±38
	3	56	131	161	348	116± 31
	4	126	90	102	318	106±11
	5	196	148	96	440	146± 29
	6	219	208	157	584	195 ±19
						121 ±22.1

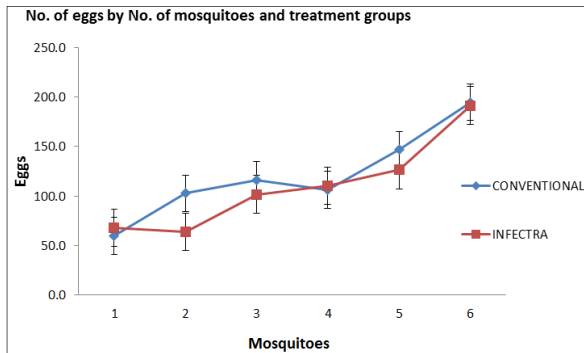


Figure 3. Number of mosquito eggs laid in the both groups of restrained mice.

### 3.2. Gametocytemia

Gametocytes were first detected on 14 dpi in animals restrained by the two methods (Fig 4). The effect of days post infection was highly significant ( $p < 0.001$ ) with Gametocytemia % increasing significantly with dpi. There was no evidence that the increase in INFECTRA®-Kit was significantly different from conventionally restrained mice and the rate of change in Gametocytemia % with dpi did not significantly vary from one treatment group to the other.  $R^2$  proportion value was 14.3%. The increase in INFECTRA®-Kit restrained mice was marked with two oscillation cycles/waves with the first cycle extending between 14 and 20 dpi and a peak of 0.44% followed by a second cycle with a peak of 1.33% at 22 dpi. The progression in the conventionally restrained mice, was marked with three cycles. The first cycle extended between 14 and 22 dpi with a peak 0.66% at 21 dpi followed by second cycle extending between 22 dpi and 25 dpi and a peak of 0.78%. This was followed by a sharp peak starting 25 dpi reaching the peak, 1.33% on 26 dpi (Figure 4).

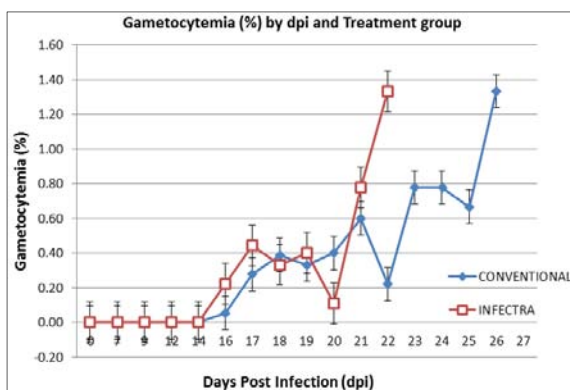


Figure 4. Changes in gametocytemia in mice INFECTRA®-Kit and conventionally restrained.

### 3.3. Oocyst count - Mosquitoes Infection (Rodent-to-Vector Transmission Assays)

All the mosquitoes fed on infected mice while restrained by the two methods picked infection successfully as was demonstrated by the production of oocysts. Mosquitoes fed on mice restrained using the INFECTRA®-Kit had a mean oocyst count of  $2.7 \pm 0.3$  (1 mosquito) which increased to a mean of  $9.3 \pm 0.3$  (6 mosquitoes). In mosquitoes fed on mice conventionally restrained, the oocyst increase was from a mean of  $3.7 \pm 0.3$  (1 mosquito) to a mean of  $8.6 \pm 0.3$  (6 mosquitoes) (Table 2, Fig 5).

Table 2. Oocyst count in mosquitoes fed on infected mice.

Mean oocyst counts per No. of mosquitoes						
No. of mosquitoes	1	2	3	4	5	6
INFECTRA®-Kit	$2.7 \pm 0.3$	$5.3 \pm 0.3$	$6.3 \pm 0.3$	$8.0 \pm 0.6$	$7.3 \pm 0.3$	$9.3 \pm 0.3$
Conventional	$3.7 \pm 0.3$	$4.3 \pm 0.3$	$6.0 \pm 0.3$	$7.7 \pm 0.3$	$9.0 \pm 0.3$	$8.6 \pm 0.3$

There was no significant difference ( $p < 0.05$ ) in oocyst production between mosquitoes infected on mice INFECTRA®-Kit and Conventional restrained. The effect of number of mosquitoes was highly significant ( $p < 0.001$ ) with oocyst counts increasing significantly as number of mosquitoes increases. The rate of increase in oocyst counts did not vary significantly between the two groups of infected mosquitoes and oocyst counts in the INFECTRA®-Kit group were insignificantly lower than in Conventional irrespective of number of mosquitoes. The  $R^2$  value was 79.99%.

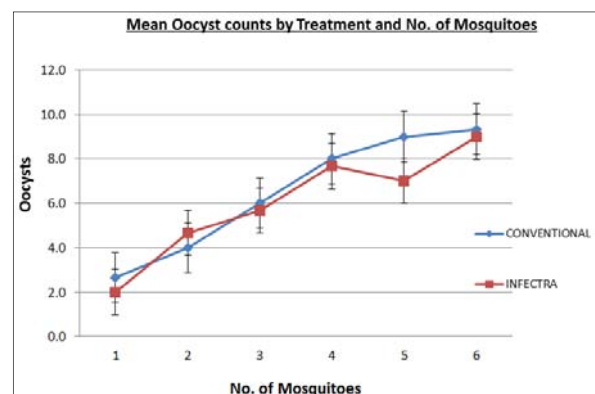


Figure 5. Oocyst counts in mosquitoes fed on infected mice INFECTRA®-Kit and conventionally restrained.

### 3.4. Mosquito-to-Mice Transmission Assays

Mice infected with the mosquitoes while restrained by the two methods developed parasitaemia (Fig. 6). The mean



prepatent period for the INFECTRA®-Kit restrained mice was  $16 \pm 0.4$  (range 14-15) days and conventionally restrained mice  $16 \pm 0.4$  (range 16-19) days respectively. The parasitaemia percentage (%) increased significantly ( $p < 0.001$ ) irrespective of the restraining method with dpi in the infected mice. Statistical analysis further showed no evidence of significant difference between the INFECTRA®-Kit and conventional methods and the interaction between dpi and the treatment was not significant implying that % change in parasitaemia with dpi did not vary from one treatment group to the other. The percentage of variance ( $R^2$ ) was 44.5%. Mice restrained using INFECTRA®-Kit demonstrated two waves of parasitaemia with a peak of 13.5% at 19 days post infection and 22% at 22 days post infection (Fig. 6). Conventionally restrained mice demonstrated three waves of parasitaemia: The first peak parasitaemia, 14% occurred at 19 days post infection followed by a second peak parasitaemia of 16.5% that occurred on day 24 and a third peak of parasitaemia of 18% that occurred on 26 days post infection (Fig. 6).

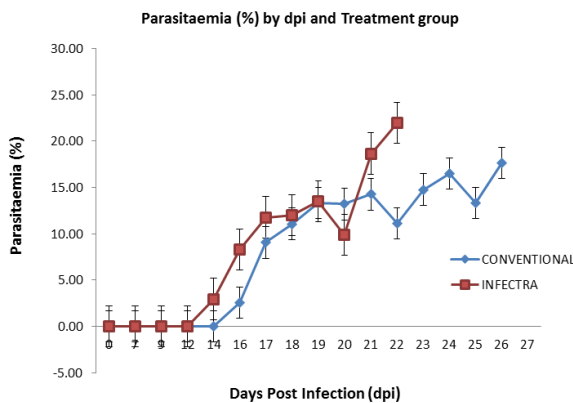


Figure 6. Parasitaemia progression in both groups of mice.

### 3.5. Body Weight (g)

A decrease in body weight was registered in INFECTRA®-Kit restrained mice starting 21 days post infection from  $21.3 \pm 1.4$ g to  $20.4 \pm 2.1$ g equivalent to 4% drop (Table 3). A similar drop of 11% from  $24.6 \pm 0.6$ g to  $21.9 \pm 1.2$ g in the conventional restrained mice was registered. Effect of dpi was highly significant implying that body weight varied significantly with change in dpi with the body weight in the control group increasing significantly ( $p < 0.01$ ) with dpi. On the other hand, body weight in the Conventional and INFECTRA®-Kit groups decreased significantly ( $p < 0.001$ ) with increase in dpi. Rate of decrease was significantly higher in the Conventional than in the INFECTRA®-Kit groups (Fig 7). The percentage of variance ( $R^2$ ) was 34%

Table 3. Mean body weight (g) with Days post infection in the groups of mice.

Restraining method	Days post infection body weight changes				
	0	7	14	21	% decline
INFECTRA®-Kit	$21.3 \pm 1.4$	$21.5 \pm 1.1$	$21.7 \pm 0.8$	$20.4 \pm 2.1$	-4
Conventional	$24.6 \pm 0.6$	$24.8 \pm 0.7$	$24.2 \pm 0.8$	$21.9 \pm 1.2$	-11
Control	$22.2 \pm 1.1$	$22.2 \pm 0.9$	$24 \pm 0.4$	$24.5 \pm 0.4$	10.4

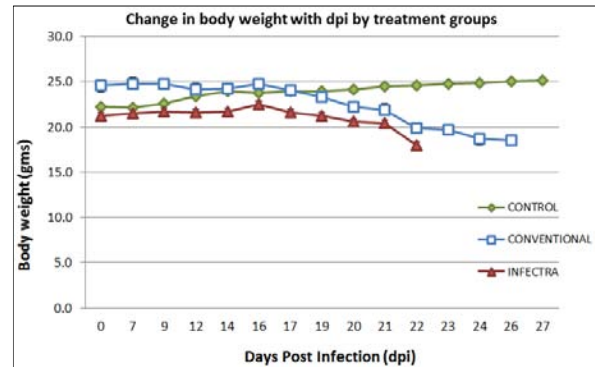


Figure 7. Changes in body weight in both groups of mice as infection progressed.

### 3.6. Survival Time

Mice infected while restrained using the two methods succumbed to death while the non-infected controls survived to post 30 dpi (Fig. 8). The INFECTRA®-Kit restrained mice had a short survival period of between 18 and 23 days post infection while those conventionally restrained had a relatively longer survival period of between 20 and 26 days post infection. The p-value associated with Wilcoxon and Log rank test of homogeneity were 0.10 and 0.9 for INFECTRA®-Kit and Conventional respectively suggesting there was no significant difference between the two methods during early and longer survival time.

#### Rank Tests for SURVIVAL TIME

Censor Variable: CENSORED

Grouping Variable: GROUP

	Chi-Square	DF	P-Value
Logrank (Mantel-Cox)	2.948	1	.0860
Breslow -Gehan-Wilcoxon	2.722	1	.0990
Tarone-Ware	2.840	1	.0919
Peto-Peto-Wilcoxon	2.727	1	.0987
Harrington-Fleming (rho = .5)	2.840	1	.0919

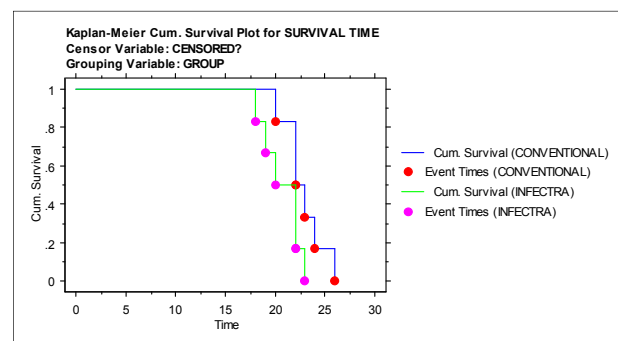


Figure 8. The survival time for mice infected while INFECTRA®-Kit and conventionally restrained.

## 4. Discussion

In this study, we tested the industrial applicability of INFECTRA®-Kit as a rodent restrainer during transmission blocking experiments using *Plasmodium berghei*. The study

was justified by the need to seek for alternative natural methods of restraining laboratory rodents during transmission blocking experiments in the laboratory and create a field set up environment that will allow natural interaction between the vector host animal without anesthesia. In this study, the applicability of the INFECTRA®-Kit in the transmission of malaria was compared with the conventional anesthesia method of restraining mice; we measured and compared the following parameters between the two restraining methods: fecundity, gametocytemia, oocyst count, parasitaemia progression, body weight changes and survival times.

The results showed that the fecundity in mosquitoes fed on INFECTRA®-Kit restrained mice was lower than in those conventionally restrained (Fig. 2, Table 1). This difference could be attributed to several factors such as the mosquito size, entomological inoculation rates (EIR) and multiple feeding which were not measured in this study. Previous studies suggests that multiple feeding increases fecundity (Thahsin & Nobuko, 2013) and smaller females require 2 or 3 blood meals to facilitate completion of the first gonotrophic cycle (Edith & Takken, 1993). It is our speculation that the state at which the mice were at the time of mosquito feeding could as well have contributed to this difference. The conventionally (anesthetized) restrained mice are normally immobile during mosquito feeding process in contrast to those INFECTRA®-Kit restrained which are not totally immobile and would as a result interrupt the continuous feeding success of the mosquitoes. However, this speculation is contradicted by results from previous studies which show that the blood meal size, oviposition rate, fecundity and post-feeding survival of mosquito vectors are significantly higher after feeding on hosts free to exhibit behavior, than those which are immobilized during feeding trials (Lyimo, *et al.*, 2012).

Gametocytes were detected in mice restrained using the two methods as has previously been observed (Dari *et al.*, 2015). The density of the gametocytes significantly increased as infection progressed which was in agreement with earlier observation (Buckling 2001). Although there was no significant difference in the gametocyte densities between mice restrained by the two methods, it was however observed that gametocytes densities were relatively higher in the INFECTRA®-Kit than in the conventionally restrained mice (Fig. 4). Gametocytes in INFECTRA®-Kit restrained mice were characterized by two waves and three waves in the conventionally restrained mice. INFECTRA®-Kit restrained mice reached the highest gametocytemia level of 1.3% at 22 dpi while the conventionally restrained mice registered the same level on 26 dpi a difference of four (4) days. Although several factors are likely to influence the appearance of gametocytes at presentation (Bousema Drakeley, 2011), the factor(s) contributing to the insignificant difference observed between the two methods of restraining and especially the effect the anesthetic drug has on the parasite virulence will require further investigation.

For the rodent-to-vector assays, mosquitoes infected by feeding on infected mice while INFECTRA®-Kit and

conventionally restrained developed oocysts which were in agreement with other similar studies (Heather & Andrew, 2004). However, there was no significant difference in the oocyst production between the two methods which therefore demonstrates the validity of the INFECTRA®-Kit in restraining mice allowing natural mosquitoes infection from an infected rodent without use of chemical anesthesia. Infected mosquito successfully transmitted infection to mice restrained by the two methods as was demonstrated by parasitaemia development and which was high in the two groups of the recipient mice. Although high levels of parasitaemia have been reported in mice infected with *P. berghei*, there was no significant difference observed in both prepatent period and parasitaemia progression in the two groups of restrained mice. The prepatent period of the INFECTRA®-Kit restrained mice was 12 days while that for the conventionally restrained mice was 14 days (Fig. 6). In both groups of mice, this was relatively longer compared to previously observed results which had prepatent period at 7 days post infection (Yamei Jin, *et al.*, 2007). Thereafter, parasitaemia in the two groups of restrained mice was characterized by high levels and this was in agreement with previous studies (Basir *et al.*, 2012). However, INFECTRA®-Kit restrained mice demonstrated relatively though not significant levels of parasitaemia compared to the conventionally restrained mice (Fig. 7).

General anesthesia has been associated with hypothermia (Lenhardt, 2010; Daniel & Sessler, 2008) which would have contributed to significantly high parasitaemia (McQuiston, 1979) in the conventionally than in the INFECTRA®-Kit restrained mice. Results from our study demonstrated the opposite. This contradiction may be attributed to the fact that the hypothermia associated with anesthesia is chemically induced and not naturally induced as was observed (McQuiston, 1979). As with the gametocytemia, further investigation on the effect of the anesthetic drug on the parasite virulence will be required. Parasitaemia progression in the INFECTRA®-Kit restrained mice was characterized by two waves of parasitaemia with the first peak 14% occurring on 19 dpi followed by the second peak 22% occurring on 22dpi. In contrast, the progression in the conventionally restrained mice was marked with three waves. The first peak 14.3% occurred on 21 dpi followed by a second wave 16.5% which occurred on 24 dpi and the third wave 17.8% occurring on 26 dpi. Results previously observed (Jann Hau and Steven, 2014) Evered and Julie Whelan, 1983), in trypanosomes and malaria infections demonstrate relationship between parasitaemia waves and disease severity with chronic/sub-acute infections characterized by several waves. From these observations we suggest that although there was no significant difference in parasitaemia progression, conventionally restrained mice presented a more sub-acute infection than the INFECTRA®-Kit restrained mice.

Mice restrained using the two methods registered a decrease in body weight as previously observed (Basir *et al.*, 2012) with INFECTRA®-Kit restrained mice reducing an

equivalent of 4% of the baseline weight at 21 dpi and the conventionally restrained mice losing an equivalent of 11% of the baseline weight at the same period whereas the control group registered an increase equivalent 10.4% (Table 3, Fig. 7). The rate of decrease was significantly more in the conventionally than with the INFECTRA<sup>®</sup>-Kit restrained mice. In deed the conventionally restrained mice registered the weight loss slightly earlier on 14 dpi to an equivalent of 1.6% decrease. The reason for this significant difference between the two restraining methods is not clear and further research will need to determine the long time effect the anesthetic drug that may have on the appetite. It has been reported that the consequences of anaesthesia may be more prolonged than previously presumed (Mcgee *et al.*, 1981) and attenuation of taste neophobia (Rondeau *et al.*, 1975) was reported as among the behavioral changes. Our results further demonstrated the average survival time for the INFECTRA<sup>®</sup>-Kit as 20±0.8 while that of the conventionally restrained mice was 22±0.8 while the control group had a survival time more than 30 days. In other studies involving trypanosomes infections (Masumu *et al.*, 2009), survival time was associated with the parasite virulence. From our results, the virulence of the parasite was maintained irrespective of the two restraining methods (Fig. 8). Using survival time as a marker of disease severity we suggest the severity of the disease was well manifested in the INFECTRA<sup>®</sup>-Kit than in the conventionally restrained mice although this manifestation was not significantly different.

## 5. Conclusion

There is sufficient evidence to conclude that results of fecundity, oocyst counts in the mosquito vectors during rodent-to-vector transmission, increased parasitemia progression and gametocytemic levels in vector-to-rodent transmission assays and succumbing to disease development and mortality determined by the prepatent period and survival times of the infected mice confirms the validity of the INFECTRA<sup>®</sup>-Kit in carrying laboratory based transmission experiments as demonstrated in a previous study (Ndung'u *et al.*, 2013). The results further demonstrated that going by the gametocyte, parasitaemia densities and survival time, INFECTRA<sup>®</sup>-Kit restrained mice presented a relatively more acute infection than the conventionally restrained leading to our suggestion that the anesthetic drug has negligible negative effect on the parasite severity. Further validation of the INFECTRA<sup>®</sup>-Kit involving other vector-borne diseases is recommended.

## Statistical Analysis

A completely randomized design was used in this study. Data was analyzed using the statistical software Genstat 14<sup>th</sup> Edition of 2011. Rodent-to-vector transmission was analyzed by log linear model. Fecundity, gametocytemia, parasitaemia and changes in body weight were analyzed by regression analysis. Survival times were analyzed Kaplan-Meier method

for determination of survival distribution function. Rank test of homogeneity were used to determine the effect of restraining method infection on survival times.

## Ethics Approval and Consent to Participate

Dates of Approval /Clearance by SSC/SERU (Quote SSC/SERU No. 2572): 2<sup>nd</sup> September, 2015.

Dates of Approval /Clearance by IACUC: 24<sup>th</sup> August, 2015.

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