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A Protocol for a Highly Consistent, High Level Production *in Vivo* of *Plasmodium falciparum* Oocysts and Sporozoites

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Abstract

Investigation of the intimate relationship between the human malaria parasite *Plasmodium falciparum* and its *Anopheles* vector requires the reliable production and isolation of successive sexual stages of the parasite from infected mosquitoes. Such an advance in propagation would benefit a range of molecular, cellular, immunochemical and transmission-blocking research studies. Parasite cultivation, mosquito rearing, infection and subsequent dissection of mosquitoes are all highly technical procedures that require both skill and experience to perform with competence. Furthermore, to produce mosquitoes of an appropriate age to infect during the short period in which parasites are viable for infection demands precise planning in order to coordinate the interacting life cycles of the parasite and vector. Here, a protocol is described for the complete development of *P. falciparum* within *Anopheles stephensi*. A very consistent, high level production *in vivo* of *P. falciparum* oocysts and sporozoites is demonstrable by dissection of the mosquito midgut and salivary glands, respectively.

Keywords

Malaria, *Plasmodium falciparum*, Cultivation, Oocyst, Sporozoite, Infectivity

1. Introduction

Malaria remains one of the major infectious diseases in the world, with an incidence of over 500 million clinical

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cases and 2 - 3 million deaths each year [1]. While it has rendered ineffectual many strategies for infection control, increasing resistance to drugs of the aetiological agent, protozoan *Plasmodium* parasites, and to insecticides of the vector, *Anopheles* mosquitoes, is prompting novel perspectives to counter both the transmission of infection and burden of the disease [2]. Of the five species of malaria that infect humans, *P. falciparum* is by far the deadliest and therefore the one against which most control measures are directed.

A major aggravating factor for the modest success of one strategy, anti-malaria vaccine design, is our inadequate understanding of the developmental processes that operate, and antigens expressed, during the differentiation of parasite stages [3], including those within the *Anopheles* vector. Immunity to sexual stages of malaria, termed transmission-blocking immunity, may operate against gametocytes in the vertebrate host and against gametes in the midgut and ookinetes, the motile form of the malaria parasite that traverses the mosquito midgut epithelium to initiate sporogony [4]. Upon maturation, each oocyst releases several thousand sporozoites into the haemocoel from which they invade the salivary glands. At this point, the sporozoites are infectious and capable of transmission to a new human host when the mosquito takes another blood meal.

In order to facilitate detailed molecular, biochemical and immunological studies of parasite progression within the invertebrate host, it is necessary to produce sufficient numbers of fully mature oocysts and sporozoites [5]. Here, we describe in detail an improved technique for the infection of *A. stephensi* with mature stage V gametocytes of *P. falciparum* and their transformation into oocysts and subsequently sporozoites.

2. Materials and Methods

2.1. Overlapping Procedures Required to Generate *A. stephensi* Mosquitoes Infected with *P. falciparum*

The successful infection of *A. stephensi* mosquitoes with *P. falciparum* involves the propagation of parasite and vector life cycles in parallel. It is a technically difficult procedure which requires considerable pre-planning and attention to detail in order to be successful. In particular, the precise timing of each stage of the process is of paramount importance to ensure a consistent supply of infected mosquitoes for the purposes of research. In our laboratory, we have found that preparing a "timeline" is a valuable organisational tool that helps identify, in short-hand form, precisely what tasks need to be performed on a day-to-day basis [6].

2.2. Safety Precautions

P. falciparum is a highly dangerous human pathogen which is classified internationally as requiring biosafety level 3 containment [7]. Hence, all appropriate health and safety measures must be carried out when maintaining this parasite in human blood *in vitro*, in keeping with local regulations and institutional guidelines. Any isolate of *P. falciparum* that is used routinely should not be resistant to standard antimalarial drugs. All work should be performed in a Class II safety cabinet and all non-essential staff should be excluded from the laboratory while work is in progress. A Howie-style laboratory coat and latex gloves should be worn at all times.

2.3. Necessity to Adhere to All Local Health and Safety Requirements

Mosquitoes capable of transmitting *P. falciparum* should be maintained within a limited access, dedicated, high security humidified insectary. It is recommended that all rooms be painted in a light colour and all cages be constructed of white materials in order to make highly visible any mosquito that may temporarily escape. A non-residual insecticide spray should also be available in the event of an emergency. Infected mosquitoes to be dissected are first immobilised temporarily, either by the preferred method of physical stunning or alternatively by light anaesthesia using chloroform. Each is then rendered permanently incapable of movement (de-winged and de-legged). Any potentially infective mosquitoes that are not dissected may be stored frozen by placing the complete container at −20˚C prior to autoclaving and disposal.

2.4. Equipment Required for Blood Feed of Mosquitoes

1) *Pooter*—a pooter is a device used to transfer mosquitoes by aspiration. A polycarbonate plastic-tubed pooter is preferred, but any toughened material is suitable provided that it is transparent. An efficient pooter can easily be modified from a 10 ml plastic pipette. This is prepared for use as follows:

A stainless steel (or nylon) fine-meshed disc of the internal dimensions of the pooter tube is inserted 2 cm from one end of a 20 - 25 cm tube. A ring may be etched in the internal surface of the tube in which the disc can sit securely. A length of clear, transparent silicon rubber tubing (approximately 30 cm) should be attached over the meshed end of the polycarbonate tube. The pooter is now ready to use, which is achieved by placing the free end of the silicon rubber tubing in the handler's mouth and aspirating by a "suck-blow" technique (similar to mouth pipetting). With practice, by this method mosquitoes may be transferred by sucking them into the open end of the polycarbonate tube and then blowing them out into a container.

2) *Mosquito container*—the ideal container should minimize the risk of mosquito escape or injury or of the handler being bitten. The container used successfully by ourselves and several other research groups is customized from a 500 ml cylindrical ice-cream container made of waxed cardboard (of the type used by Ben & Jerry's and Häagen-Dazs). This is prepared for use as follows:

A circular piece of filter paper (Whatman no. 1, or similar) is cut to fit the internal dimensions of the container and taped to its floor in order to soak up any spillage that may occur (from nutrient feed, diuresis drops, etc.) when in use.

A 3 cm square is cut in the side and the hole covered with two layers of dental latex (HCM rubber dental dam or similar, available from local dental supplies distributors) into which two 1 cm cuts are made, one of which is then placed perpendicular to the other. Both squares of latex should be held firmly in place with electrician's insulating tape. This results in a cross-shaped entry port through which, with the aid of a pooter, mosquitoes can be safely introduced or removed. To complete the container, a double layer of bridal veil netting is secured over the mouth with elastic bands and insulating tape. Other than during an actual blood feed, containers should be kept inside a gauze-covered cage that acts as a secondary security barrier.

3) *Blood feeder*—aglass membrane feeder is used to deliver the infectious blood meal to the mosquitoes. Blood feeders consist of a water-jacketed chamber into which the infectious blood feed can be introduced. Ours are custom-made by a professional glass blower (contact details upon request). This is prepared for feeding use as follows:

The blood feed chamber is covered with a Baudrouch membrane derived from bovine intestine (Joseph Long Inc., New Jersey). The membrane is wetted with tap water to ensure that, when dry, it is taut. This encourages the mosquitoes to feed. If washed and dried thoroughly after each use, a membrane can be used on up to six occasions before it loses its integrity. In fact, in our experience, previously used membranes are preferred to new membranes because their use results in a greater proportion of mosquitoes taking a blood meal. The membrane should be held in place with two elastic bands. Tap water at 38˚C is passed through the water jacket via a recirculating pump (water inlet via the lower tube, outlet via the higher tube).

2.5. Rearing of *A. stephensi* Mosquitoes

Each stock colony of *A. stephensi* should be housed in a gauze-covered, plastic-covered wire-framed cage. We use cages of frame dimensions 30 cm^3, which are covered with mosquito netting or "bridal veil" netting to incorporate a 15 cm front sleeve. Cages are kept at 26.0˚C ± 0.5˚C and 80.0% ± 2.0% relative humidity. All colonies are maintained on a reverse light cycle with 12 h artificial lighting from 2000 to 0800 h. This enables all manipulations to be performed in keeping with the nocturnal activity of the mosquito during normal working hours.

Adult female mosquitoes should be fed twice a week on uninfected rodent blood. We routinely feed directly by bite of naive outbred laboratory mice, but rats or guinea pigs are also suitable. The feeder mouse is anaesthetized by intraperitoneal injection of 0.1 ml per 10 g body weight of 10% Hypnorm (Janssen Pharmaceutica) and 20% Valium (Roche) in sterile distilled water. The sedated mouse is placed abdomen-down, with the four limbs stretched out, on top of the cage in which the mosquitoes to be fed are kept, and feeding allowed for 20 min. The abdomen may be shaved of fur to facilitate access for feeding. Mice under terminal anaesthesia should be used in accordance with project and personal licences issued by local regulatory authorities and following institutional guidelines. Where a local regulatory authority permits rodents to be reused for the purposes of mosquito feeding, they can be allowed to recover from the anaesthetic. Alternatively, and with equally effective results, a mouse may be exsanguinated under terminal anaesthesia and the blood decanted into a blood feed chamber and this used (without the addition of anticoagulant) for immediate membrane feeding to mosquitoes.

Eggs are laid by fed female mosquitoes 2 - 3 d after a blood feed. A small bowl lined with filter paper (Whatman no. 1, or similar) and filled with tap water should be placed on the floor of the stock cage overnight.

The following morning, the bowl is removed to another cage and covered with a square of transparent Perspex until the eggs begin to hatch (within 2 d).

Hatched larvae are reared in clean plastic washing-up bowls (preferably coloured white) containing 7 - 8 cm of water. The contents of the hatching bowl should be transferred carefully into a larval rearing bowl (approximately 300 larvae per bowl) and the filter paper discarded. The water must not contain large amounts of chlorine so it may be necessary to allow the local tap water to stand for a minimum of 24 h before use to allow any chlorine to dissipate ("chlorine-free" water). The continuous maintenance of a reservoir of pre-prepared chlorine-free water is recommended. Alternatively, water can be boiled before use or bottled water can be used. Whatever the source and treatment of the water, it must be at room temperature before eggs are added.

Anopheles mosquito larvae are primarily surface feeders and must be provided with a form of food that floats on water. We use koi carp pellets (Wardley Corporation premium koi staple, or a suitable alternative supplied by a local aquarist). Baby weaning formulation milk powder ("Farex" or similar) may also be used but should to be finely sieved before sprinkling on the water. Larvae are fed morning and evening with fresh pellets (a "little but often" regime). Any surplus "old" food should be removed and discarded. In this way, the larvae receive sufficient food, but not so much that the bowl becomes contaminated with uneaten food. The density of larvae should be reduced after 2 - 3 d. By keeping larvae numbers between 200 - 250 per bowl, large well-nourished mosquitoes are produced.

Larvae should pupate 6 - 8 d post emergence from eggs if kept at 26˚C and 80% relative humidity. Pupation can be delayed by 2 - 3 d by reducing the ambient temperature by 1˚C - 2˚C. Surplus larvae are killed by immersion in hot water; they should not be discarded live down the sink. Pupae are collected and placed in a bowl of chlorine-free water. A filter-paper cone should be prepared, 2.5 cm cut off the point and the now topless cone placed over the bowl. This system allows emergence of adult mosquitoes (2 - 3 d post-pupation), but also deters emergent mosquitoes from flying back into the bowl and drowning and keeps fed females from laying eggs in the pupae bowl. This is placed back in the stock cage.

A solution of 5% glucose (Sigma) and 0.05% *p*-aminobenzoic acid (PABA; Sigma) in chlorine-free water is provided for all adult mosquitoes. This nutrient solution should be sterilized by passing through a 0.22 μm filter (Sterilin or similar) before soaking a cotton wool pad and placing it in the cage. The pad should be moist but not dripping as this leads rapidly to fungal contamination, and should be changed daily.

2.6. On the Day before the Feed

The mosquito container(s) should be prepared and 4 - 6 d-old virgin female mosquitoes (*i.e.* 5 - 7 d post-emergence from pupae on the day of the feed) placed into each, with the aid of a pooter. A suitable number of mosquitoes are added for the volume of the vessel to be used; for a 500 ml cylindrical container, add between 100 - 250 mosquitoes. Up to 20 mosquitoes can be accommodated comfortably within a pooter at any one time to enable counting during the transfer procedure. It is important to handle the mosquitoes with care so as not to damage them.

Mosquitoes are tested for their readiness to blood-feed by evaluating their attempt to feed on a warmed, water-filled, tissue culture flask placed on top of the gauze-covered stock cage in which they are housed. If the pooter is used for transfer to the feed container while the culture flask is in place, those mosquitoes that are attracted by the warm object can be positively selected. This procedure acts not only to select older individuals more eager for blood among a population of slightly mixed ages (due to variation in the timing of emergence from pupation) but also to segregate female from male mosquitoes. Only females are attracted by the warmed flask, as its temperature mimics that of a human (from which they require blood in order to lay eggs); male mosquitoes do not blood-feed and are ambivalent to the presence of the flask.

Mosquitoes are fed on a cotton wool pad soaked in 5% glucose/0.05% PABA which is placed on top of the mosquito container. The pad is removed 12 h before the blood feed. This starvation step encourages a greater number of mosquitoes—usually the vast majority—to subsequently feed to engorgement on blood.

2.7. On the Day of the Feed

A Baudrouch membrane is placed on the blood feeder, wetted and allowed to air-dry (approximately 20 min). Once the membrane is dry, the water pump is switched on and the water jacket allowed to warm up to 38˚C (approximately 60 min).

Preparation of human erythrocytes and pooled serum, and effective propagation of *P. falciparum* gametocytes *in vitro* are described in detail elsewhere [8]. Fresh group O, Rhesus group-positive whole blood should be washed and warmed to 37˚C. Likewise, group O, Rhesus group-positive serum is warmed to 37˚C. *P. falciparum* to be used for the feed should be taken from 14 - 17 d-old cultures which contain mature, stage V gametocytes. Since male gametocytes mature more rapidly than do females, in practice it is best to use either: 1) exclusively cultures that are 17 d post-subculture, which should contain a sufficient number of gametocytes of both sexes; or 2) a preparation of blood of different ages (such as a 1:1 mix of 14 d and 16 d-old cultures), which will show a sex ratio bias in favour of male or female parasites, respectively. In our experience, artificially mixed preparations generally achieve better rates of infectivity.

It is essential to perform the following procedures entirely at 37˚C (including using pre-warmed plastic ware and centrifuge buckets) and as rapidly as safe handling and good microbiological practice permits. This is in order to minimize the possibility of gametocytes committing to activation/exflagellation before the blood meal is taken up by the mosquito [9] (see below).

Infectious cultures should be transferred to centrifuge tube(s) and spun at 800 × g for 2 min. After the supernatant is removed and discarded, the volume of the remaining pellet, containing packed parasitized erythrocytes, is measured. To this is added an equal volume of serum and three wash steps performed. The volume of infective blood/serum is then made up to that of the blood feeder to be used (1 - 2 ml) by diluting the suspension up to 3 × volume with a mixture of 1:1 washed human erythrocytes/serum, mixing gently but thoroughly. Blood is diluted in this manner because: a) better infection rates are achieved using blood that contains a high but submaximal gametocytaemia; b) addition of fresh blood/serum to the 17 d-old cultured parasitized erythrocytes promotes mosquito feeding, in terms of both extending the feeding time of individual mosquitoes (qualitative) and increasing the number that feed (quantitative).

Once prepared, the diluted infective blood is transferred rapidly into the blood feeder using a pre-warmed syringe fitted with a blunt-ended needle, ensuring that there are no air bubbles present. A mosquito container should be removed from its outer cage and placed underneath the blood feeder, ensuring that the feeder and the netting are in intimate contact, thereby minimizing the risk of escape of fed (and therefore potentially infectious) mosquitoes. Any artificial lighting should be switched off and the mosquitoes allowed feeding undisturbed for 12 - 15 min. We recommend that the high security insectary in which the mosquitoes are held be without windows; this enables feeding to take place in the dark, mimicking feeding behaviour in the wild.

To finish feeding, the mosquito container is removed from underneath the blood feeder and returned to a gauze-covered cage. Most mosquitoes that have been starved of nutrients for the previous 12 h will feed to engorgement within a few minutes. These will have a very distended, red abdomen and can be distinguished easily from mosquitoes that have not fed. If the mosquitoes have fed well, the floor of the container will be covered with red splashes of fluid passed by diuresis. Depending on the ease with which the pooter can be manipulated within the container, it may be possible at this stage to remove individual unfed mosquitoes. The time during which the blood feed is taking place may be used to examine any leftover infective blood/serum mix that was not added to the membrane feeder for gametocyte activation and/or exflagellation.

Exflagellation is the explosive production of male gametes of the malaria parasite, which happens in the mosquito midgut within a few minutes of a blood meal. This phenomenon also occurs spontaneously *in vitro* and thus may be observed in cultures of fresh parasitized blood under the light microscope (×1000 magnification under oil immersion). It is controlled *in vitro* solely by the change from 37˚C to the ambient laboratory temperature, the pH rise this brings being mediated by a fall in CO_2 tension as the blood equilibrates with the atmosphere. Viewing of gametogenesis (typically between 10 - 25 min after removal of a blood culture from the 37˚C incubator) is a strong indicator that oocysts and sporozoites will be produced following a blood feed. However, in our experience, observable exflagellation is not a prerequisite for successful mosquito infectivity.

2.8. On Days Following the Feed

Subsequent to a blood feed, mosquitoes should be fed daily on a 5% glucose/0.05% PABA diet, as previously described. Delaying the start of feeding by 24 h post blood feed enriches for blood-fed mosquitoes as the vast majority of “die off” mosquitoes under these conditions are those that did not blood-feed and therefore were starved of nutrients for at least 36 h. As feeding on blood promotes mosquito survival under these conditions, this strengthens the validity of oocyst and sporozoite counts that are performed.

Mosquitoes may be dissected for the presence of oocysts at 10 - 12 d post blood feed. Dissection for sporozoites may take place from 14 d post blood feed. The number of live mosquitoes in each container at the time of dissection must be verified and recorded by two people independently (usually by observing carefully the pootering process). The total number of mosquitoes either dissected or undissected at the end of the process should tally with the records made. The protocol for dissections is described below.

2.9. Dissection of Mosquito Midgut to Determine the Presence of Oocysts

For the detection of *P. falciparum* oocysts, mosquitoes should be dissected 10 - 12 d post infective blood feed. Mosquitoes are removed from the container via a pooter, the end of which is immediately plugged with cotton wool and sealed with masking tape to prevent escape. Mosquitoes may be collected one at a time, but up to 5 mosquitoes can be held in the cavity of the pooter without compromising handler safety. The pooter is struck firmly against the palm of the hand several times to knock out the mosquitoes for a sufficient period of time to allow their safe removal onto a dissection board. In order to stop each mosquito from flying, and also as an aid to dissection of the body, its legs and one wing are removed with a scalpel blade.

De-winged and de-legged mosquitoes should be placed in a watch glass containing dissection medium (PBS + 0.0001% FCS). Using a pair of fine forceps, each mosquito is picked up by its one remaining wing and dipped into 70% ethanol to ensure both sterility and killing of the mosquito. Individual mosquitoes are mounted on glass microscope slides onto which a drop of dissection medium has been placed, and the remaining wing removed. Each mosquito should be dissected under a binocular light microscope using a ×40 objective (total magnification ×400).

Dissection is started by separating the abdomen from the rest of the mosquito. The gut is removed by holding the anterior of the abdomen with one dissecting needle while at the same time making a small cut with a second needle in the tegument on each side of the seventh abdominal segment. Using the second needle, the apex of the abdomen is pulled gently until the gut and Malpighian tubules are exposed. The alimentary canal is severed sufficiently far forward to bring away (and discard) all of the foregut except for the section immediately proximal to the midgut. The remainder of the gut is anchored by placing the point of one needle on the posterior section of foregut. Using the other needle, a cut is made through the alimentary canal at the junction of the midgut and the hindgut, simultaneously severing the Malpighian tubules. The hindgut and Malpighian tubules may be discarded, leaving only the midgut on the slide. When a particularly rapid dissection is required (with least chance of disruption to the oocysts), once the gut has emerged completely from the abdomen, it is acceptable to cut off and remove the hindgut only, leaving the midgut exposed but with the foregut and oesophagus still attached.

In order to count the oocysts contained in a dissected midgut, it should be picked up using a needle and transferred to a fresh slide onto which has been placed a drop of dissecting medium. A coverslip is lowered gently onto the moistened midgut, which then can be viewed for the presence of oocysts. The volume of medium bathing the preparation is important as too little will cause the midgut to rupture (so releasing oocysts and thereby preventing their examination *in situ*) and too much will prevent adequate flattening of the midgut for optimal viewing. Excess medium may be drawn off by holding a filter paper to the edge of the slide.

Examination should take place under ×400 magnification with reduced light, when oocysts, if present, are easily identified as circular refractive bodies on the gut wall of the dissected mosquito (**Figure 1** & **Figure 2**).

2.10. Dissection of Mosquito Salivary Glands to Determine the Presence of Sporozoites

Freshly dissected salivary glands containing *P. falciparum* sporozoites should be considered as potentially infectious and so must be handled with appropriate caution.

Sporozoites may first be observed in the salivary glands 14 - 17 d post infective blood feed. The junction of the mosquito head and thorax contains a pair of glands, each of which is tri-lobed (or very rarely four-lobed). The initial preparation steps of de-winging and de-legging the mosquito are as described above for oocyst dissection.

A dissecting needle is placed gently on the thorax of the mosquito, just below the region where the salivary glands lie. A second needle is then placed on the ‘neck’ of the mosquito (apex of head and thorax) without cutting it; a gentle pulling action is sufficient to detach the head. This should expose the salivary glands which it is now possible to separate from the head. If the salivary glands are not apparent, they should emerge upon pressing with a needle again on the thorax, and can be detached. The dissected glands in a small volume of dissection

Figure 1. Two oocysts, dissected from the outer wall of the *Anopheles stephensi* midgut, 10 days post infection of the mosquito (wet mount, ×400 magnification).

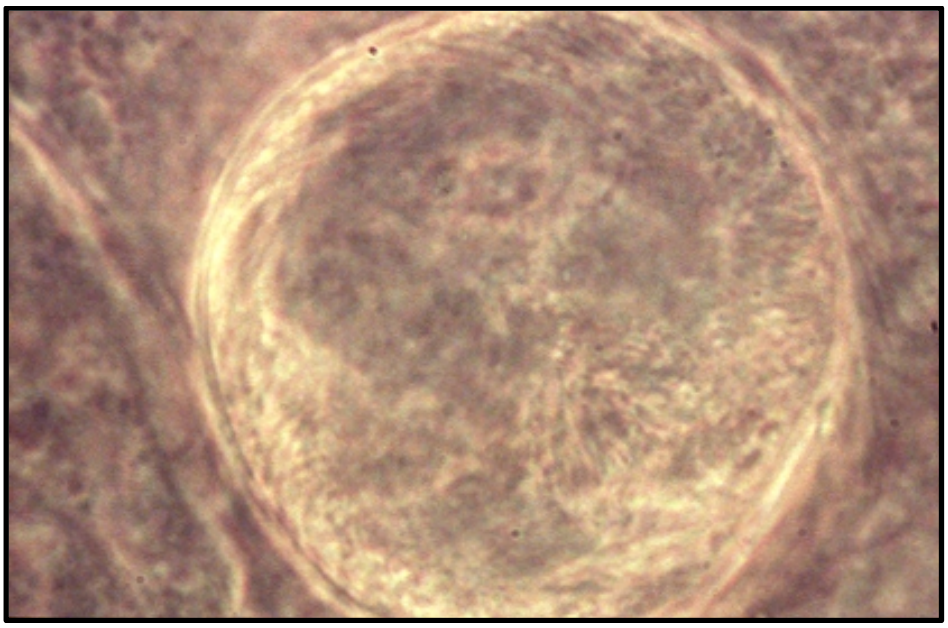

Figure 2. Single oocyst, dissected from the outer wall of the *Anopheles stephensi* midgut, 10 days post infection of the mosquito (wet mount, ×1000 magnification under oil immersion).

medium should be transferred into a 0.5 ml microcentrifuge tube using a 100 - 200 µl Gilson pipette, and this kept on ice.

2.11. Liberation of Sporozoites from Salivary Glands and Estimation of Level of Infection

The microcentrifuge tube containing the salivary glands should be covered with Parafilm to reduce the chance of leakage. Sporozoites are released by gently rupturing the glands using a laboratory benchtop whirlimixer set to maximum for 3 min or by vigorous pipetting using a 200 µl Gilson pipette. Once liberated from the salivary glands, the sporozoite yield can be estimated by counting using a haemocytometer. 10 µl of the sporozoite sus-

pension is placed into the haemocytometer chamber and a phase-contrast microscope set to ×200 to ×400 magnification used to visualize the motile parasites. The number of sporozoites present in a 4 × 4 grid is counted relates to the number of sporozoites per mm^2 $\times 10^4$.

If a qualitative examination for sporozoites is all that is required, salivary glands may be transferred directly to a glass microscope slide. A coverslip is lowered carefully onto the moistened glands and tapped gently to disrupt the glands and thereby release any sporozoites present. These should be identified easily as comma-shaped, motile bodies (**Figure 3**).

Use of immersion oil for microscopical examination (×1000 magnification) can cause gradual fading of Giemsa-stained slides. Thus, if a permanent record is required it is advisable to mount a camera to the microscope so that images may be captured.

3. Results

By following this methodology, high rates of infectivity may be anticipated following feeding of mosquitoes to engorgement. In our experience, the number of oocysts recovered per infected mosquito varies between 1 and >200, with an average of approximately 30 [10]. Mosquitoes that are not infected, as determined by a lack of oocysts, are usually those that did not blood-feed and will not contain eggs. Very occasionally, an infected mosquito (with oocysts) that has no eggs may also be seen. The number of sporozoites recovered per infected mosquito varies between 4000 - 30,000, with an average of approximately 15,000.

4. Conclusion

The excellent infectivity of *P. falciparum* for *A. stephensi* that may be routinely achieved using the protocol described here provides a powerful tool to facilitate the conduct of a wide range of molecular, biochemical, immunological and vaccine studies of the sexual stages of *P. falciparum in vivo*.

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The authors have declared no competing interests.

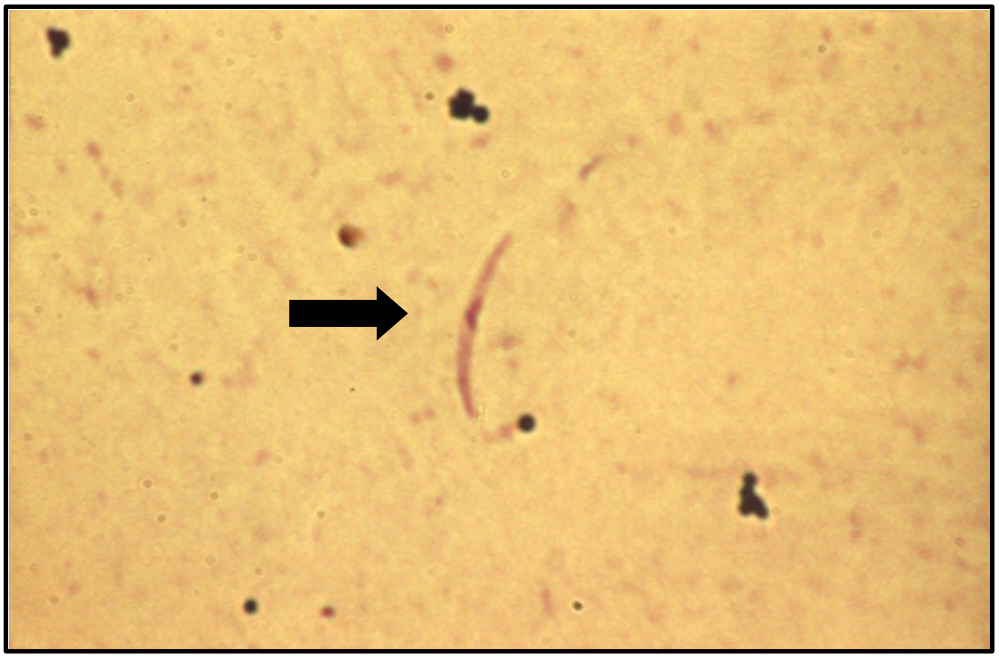

Figure 3. Isolated bow-shaped sporozoite (arrowed), dissected from the salivary glands of *Anopheles stephensi*, 17 days post infection of the mosquito (wet mount, ×1000 magnification under oil immersion).

Authors' Contributions

M. Looker performed gametocyte cultivation, mosquito production, infectivity studies and dissections. A. W. Taylor-Robinson supervised execution of the work and helped with *in vitro* parasite cultivation and dissection of mosquitoes. Both authors conceived the design of the study, processed and analysed the data, prepared the manuscript and approved the final version.

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