Safety and feasibility of apheresis to harvest and concentrate parasites from subjects with induced blood stage Plasmodium vivax infection

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Abstract

Background In the absence of a method to culture Plasmodium vivax , the only way to source parasites is ex vivo . This hampers many aspects of P. vivax research. We assessed the safety of apheresis, a method for selective removal of specific components of blood as a means of extracting and concentrating P. vivax parasites.

Methods An iterative approach was employed across four non-immune healthy human subjects in separate cohorts. All four subjects were inoculated with ~564 blood stage P. vivax parasites (HMP013- Pv) and subjected to apheresis 10 to 11 days later. Blood samples collected during apheresis were tested for the presence and concentration of P. vivax parasites by microscopy, flow cytometry, 18S rDNA qPCR for total parasites, and pvs2 5 qRT-PCR for female gametocyte transcripts. Safety was determined by monitoring adverse events. Malaria transmission to mosquitoes was assessed by membrane feeding assays.

Results There were no serious adverse events and no significant safety concerns. Apheresis concentrated asexual parasites by up to 4.9-fold (range: 0.9 - 4.9-fold) and gametocytes by up to 1.45-fold (range: 0.38 - 1.45-fold) compared to pre-apheresis densities. No single haematocrit layer contained >40% of all the recovered P. vivax asexual parasites. Ex vivo concentration of parasites by percoll gradient centrifugation of whole blood achieved greater concentration of gametocytes than apheresis. Mosquito transmission was enhanced by up to 5-fold in a single apheresis sample compared to pre-apheresis.

Conclusion The modest level of parasite concentration suggests that the use of apheresis may not be an ideal method for harvesting P. vivax parasites.

Trial Registration Australia New Zealand Clinical Trials Registry (ANZCTR) Trial ID: ACTRN12617001502325 registered on 19th October 2017. https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=373812

Background

Plasmodium falciparum is the most prevalent malaria parasite in Africa. However, Plasmodium vivax has a wider geographical distribution. In 2018, there were approximately 75 million cases of malaria due to P. vivax, accounting for 50% of cases in South East Asia and 75% of cases in the Americas(1).

In the absence of a method for continuous in vitro culture of P. vivax, parasites are usually sourced ex vivo from infected humans. This limits many aspects of the study of P. vivax research, including the development of interventions to control and eliminate P. vivax, such as diagnostics, drugs and vaccines. For example, a reliable source of P. vivax sporozoites is required to test and develop new drugs targeting the dormant liver-stage parasites – the hypnozoites. Currently this entails an expensive, logistically complex and unreliable process of sourcing P. vivax-infected mosquitoes from endemic areas. In addition to the practical issues, parasites sourced in this way are not genetically homogenous. Thus, experiments are subject to possible effects of strain variability.

Experimental infection of human subjects with malaria termed volunteer infection studies (VIS) or controlled human malaria infection (CHMI) studies are increasingly being used for drug and vaccine development(2–7).

Infections can be induced by mosquito bite, injection of sporozoites or blood-stage parasites. The latter is used in the induced blood stage malaria (IBSM) model, where healthy subjects are injected with Plasmodium infected red blood cells. The IBSM model is being increasingly used for clinical studies of P. vivax(7–9).

Recently we have shown successful transmission of P. vivax from healthy subjects to Anopheles stephensi mosquitoes(9, 10). In this study sporozoites were harvested from the infected mosquitoes that were able to infect human hepatocytes in vitro(9). Although this system offers the potential to study the biology of P. vivax malaria transmission and liver stage parasites, it is not a sustainable large scale source of sporozoites for downstream work.

Apheresis is the removal of a specific component of an individual's blood. Currently, centrifugal apheresis is the preferred method whereby blood components are separated based on buoyancy. Computer-controlled automated apheresis systems undertake continuous removal, separation of the target component, and then return the remaining blood to the individual(11). Automated erythrocytapheresis, also known as red cell exchange (RCE) has been used in the past for treatment of severe P. falciparum malaria with the rationale of reducing the parasitized red blood cell concentration by replacing Plasmodium-infected red blood cells with normal donor red blood cells(12). However, the rapid parasite clearance resulting from artesunate therapy has negated the need for RCE as a treatment for severe malaria(13, 14).

Here we report the results of a P. vivax clinical trial where we aimed to assess the use of apheresis as a means to harvest and concentrate all blood stages of P. vivax, including gametocytes, from human subjects experimentally infected with blood-stage P. vivax parasites.

Methods

Study Design

The study presented here is a Phase 1 exploratory study that was conducted in four sequential single subject cohorts (ANZCTR Trial ID: ACTRN12617001502325) and performed at Q-Pharm Pty Ltd, Brisbane, Australia and the Apheresis Unit at the Royal Brisbane and Women's Hospital (RBWH), Australia between October 2017 and May 2019. The primary objective of the study was to determine the safety of the P. vivax infection in healthy subjects following inoculation with blood-stage parasites, and the safety of apheresis for collection of P. vivax parasites from experimentally infected subjects. Secondary objectives were to assess the feasibility of apheresis as a method of harvesting, concentrating and subsequently cryopreserving P. vivax parasites from healthy subjects. Exploratory objectives were to evaluate the potential for apheresis to be used as a method for producing a P. vivax human malaria parasite bank, to evaluate the transmission of P. vivax gametocytes to mosquitoes and to collect and store plasma and peripheral blood mononuclear cells harvested using apheresis.

Specific modifications to the study protocol, such as the apheresis procedure, were required between subjects in an attempt to optimise the procedure and meet the objectives. All changes made between subjects were based on the findings from previous subjects.

Study subjects

Healthy adult males and females, aged between 18 and 55 years who met all inclusion criteria and none of the exclusion criteria were eligible for participation. Subjects were required to be malaria-naïve, Duffy blood group positive and have blood type O. Female subjects had to be Rh(D) positive. All subjects had to be available for a safety follow up period of three months. A full list of the inclusion/exclusion criteria for this study is included in the study protocol located in Additional File 1 .

Study Conduct

Pre-clinical component

A pre-clinical experiment was conducted prior to the inoculation of subjects in order to confirm the feasibility of harvesting Plasmodium parasites using apheresis.

The P. falciparum NF54 clone was used in these experiments (15) due to limited availability of P. vivax parasites. P. falciparum infected red blood cells (17.6 ml; 16 ml blood with 0.1% asexual parasitemia and 1.67 ml blood with 0.01% gametocytaemia) were added to 450 ml of fresh venous whole blood and subjected to ex vivo apheresis. Samples were collected from the 1%, 2%, 3%, 5% and 7% haematocrit (HCT) layers as determined by visualising the colour saturation of the apheresis product. An automated haematology analyser (Sysmex XN-3000; Sysmex UK) was used retrospectively to confirm the HCT of samples collected during apheresis. Presence of parasites was assessed in each layer by 18S qPCR(16) and microscopy.

Clinical component

Following intravenous injection of P. vivax parasites (day 0), subjects were monitored by daily telephone calls until day 4, when subjects visited the clinical unit daily until the day of apheresis. Subjects were monitored for adverse events (AEs), signs and symptoms of malaria infection, and blood was collected for 18S qPCR measurement of parasitaemia. The severity of AEs were determined by the common terminology of clinical trial adverse events (CTCAE) v. 4.03 (17).

The threshold for commencement of apheresis and treatment with artemether-lumefantrine was within 24 hours of a parasitemia > 20,000 parasites/mL, or the Malaria Clinical Score reaching > 6 (10), or at the Investigator's discretion. The morning that this threshold was reached (anticipated based on previous studies to be Day 10 or 11(9), subjects were admitted to the clinical unit (Q-Pharm) for initial safety assessments before being escorted to the Apheresis Unit at RBWH by Q-Pharm staff. The Apheresis Unit is located in the Haematology Department at RBWH where patients are subject to donor or therapeutic apheresis. At the Apheresis Unit the subjects underwent the apheresis procedure as per the Standard Operating Procedure (Additional Files 2 to 5) whilst being supervised by the apheresis specialist nurse and under the supervision of the responsible clinical haematologist (GK). The same apheresis nurse performed the apheresis procedure for all four subjects. The apheresis procedure lasted 1–4 hours. Subjects were then escorted back to the clinical unit and began treatment with artemether-lumefantrine (Riamet®, Novartis Pharmaceuticals Australia Pty Ltd). Treatment consisted of six doses of 4 tablets at 12 hourly intervals (each tablet contains 20 mg artemether and 120 mg of lumefantrine). Subjects remained confined within the clinical unit for 48–72 hours for safety monitoring. Following release from confinement, subjects attended protocol specified visits until three months post-treatment to monitor for signs of parasite recrudescence and to assess late safety signals. A schematic of the study design is shown in Additional File 6 Fig. 1.

This study used an iterative adaptive design approach where subject safety and outcome data were analysed after each subject and modifications made to improve the chances of meeting the exploratory objectives in the subsequent subject. A summary of the changes instituted is shown in Table 1.

Table 1. Summary of main study design differences between subjects

	Subject 1	Subject 2	Subject 3	Subject 4
sis ure	CMNC	CMNC	CMNC	Red cell depletion followed by CMNC on red cell depletion product
vers sampled	1%, 2%, 3%, 5%, 7%	1%, 2%, 3%, 5%, 7%	0.5 %, 1%, 2%, 3%, 5%, 7%, 11% 2-3%, 5-7%, 1-7%	From the primary apheresis (HCT): Intermediate (64%) From the secondary apheresis (HCT): Final (3%) Spare (5%) Waste (42%)
sis timepoint	10	10	11 PM	11 AM
o feeding amples	Pre-apheresis (with-percoll enrichment), 1%, 2%, 3% HCT layers	Pre-apheresis (with-percoll enrichment)	Pre-apheresis (with and without-percoll enrichment)	Pre-apheresis (without-percoll enrichment), Intermediate, Final, Waste
lood:citrate ring sis	15:1	8:1	8:1	13:1
added to sis collection	No	Yes	Yes	Yes
al es*	No	No	Yes	Yes

CMNC; continuous mononuclear cell collection, HCT; haematocrit. Protocols for subjects 1 to 4 and all experiments can be found in Additional Files 7 to 10. *Biological duplicates involved repeat 18S qPCR testing from two separate blood samples from each HCT layer collected using apheresis.

Malaria Challenge Agent

The P. vivax human malaria parasite (HMP) bank HMP013 was derived from blood group O rhesus positive blood donated from a returned traveller from India who presented with clinical manifestations of malaria(9). The inoculum was prepared as previously described(18).

Measurement of Parasitaemia by qPCR

Parasitaemia was quantified using 18S qPCR targeting the highly conserved Plasmodium 18S ribosomal RNA gene(16, 19). Quantitative reverse transcriptase PCR (qRT-PCR) assays were used to measure gametocytemia

with assays targeting the P. falciparum pfs25 (female) and pfMGET (male) gametocyte mRNA transcripts(20) and P. vivax pvs25 gametocyte mRNA transcripts(21).

Flow Cytometry

Flow cytometry was performed to characterise cell populations present in samples collected during the apheresis process. A combination of stains and antibodies were used to identify cells containing DNA/RNA (SYBR Green I), white blood cells (WBCs) (CD45 antibody) and/or reticulocytes (CD71 antibody). Samples from subject 1 were stained with SYBR Green I (Molecular Probes) ; samples from subjects 2 and 3 were stained with SYBR Green I and CD45-PacificBlue; and samples from subject 4 were stained with SYBR Green I, CD45-Pacific Blue and/or CD71-APC. Samples were kept on ice or at 4–8ºC until analysed by flow cytometry.

SYBR Green I staining

A volume of 2.5 µl or 1×10^6 cells from each sample was stained with 30–50 µl of SYBR Green I at 10x for 30 minutes in the dark. After incubation, 200 µl of FACS buffer (2% fetal bovine serum in phosphate buffered saline) was added.

Antibody staining

Approximately 1×10^6 cells were stained with 5–10 µg/ml of CD45-Pacific Blue or 2.5 µl of CD71 stock solution for 30 minutes at 4–8ºC in the dark. Cells were washed twice with PBS by centrifugation at 1455 xg for 4 minutes, at 4ºC. After the last wash 200 µl of FACS buffer was added to the cells.

Double staining with SYBR Green I and CD45-Pacific Blue: A volume of 30 µl of SYBR Green I at 10x was added to pelleted cells that were previously stained with CD45-Pacific Blue (as mentioned above) for 30 minutes at 4–8ºC, in the dark. After incubation a volume of 200 µl of FACS buffer was added.

Triple staining with SYBR Green I, CD45-Pacific Blue and CD71-APC: Approximately 1×10^6 cells were stained with 10 µg/ml of CD45-Pacific Blue, 2.5 µl of CD71 stock solution and 30 µl of SYBR Green I at 10x. Samples were incubated for 30 minutes in the fridge (4–8ºC) in the dark. Cells were washed twice with PBS by centrifugation at 1455 xg for 4 minutes, at 4ºC. After incubation a volume of 200 µl of FACS buffer was added.

Flow cytometry analysis

Samples from subjects 1, 2 and 3 were acquired on a FACS CANTO II (BD Biosciences), using the 488 nm and 405 nm lasers. SYBR Green I positive cells were detected using a 530/30 nm band-pass filter and CD45-Pacific Blue positive cells were detected using a 450/50 nm band-pass filter. Samples from subject 4were acquired on a LSR FORTESSA (BD Biosciences), using the 488 nm, 640 nm and 405 nm lasers. SYBR Green I positive cells were detected using a 530/30 nm filter, CD45-Pacific Blue positive cells were detected using a 450/50 nm filter and CD71-APC positive cells were detected using a 670/14 nm filter. Flow cytometry data was analysed using FlowJo® software (version 10.8, Tree Star Inc, Oregon, USA).

Microscopy

Thick and thin smears were stained with Giemsa and examined under a 100 × oil immersion objective by level 1 or 2 WHO certified malaria microscopists. Apheresis samples were expected to have a significantly different composition in terms of proportions of RBCs and WBCs when compared to whole blood (e.g. RBCs make up 1% and approximately 46% of 1% HCT and whole blood samples respectively). As such, standard parasitemia measures by microscopy were not feasible. It was decided that the expert microscopists would estimate parasitemia based on sample composition.

Mosquito Feeding Assays

Transmissibility of pre-apheresis samples and post-apheresis samples to Anopheles stephensi mosquitoes was evaluated using membrane feeding assays (MFA)(9, 22). For enriched MFA, gametocytes present in 80 mL of whole blood (pre-apheresis) were enriched in 70% percoll gradient. For direct MFA (DMFA), 650 µL of pellet from whole blood (pre-apheresis) or from each apheresis sample was reconstituted to 50% haematocrit with malaria naïve AB + serum. Infection in midguts was assessed by qPCR(23) 8 days after the feeding assays. For logistic reasons, enriched MFA was not carried out in subject 4. Following consideration of gametocyte levels, DMFA was not carried out in subject 2.

Apheresis Procedures

Apheresis was carried out using a Spectra Optia v11.3 apheresis system (Terumo BCT, Inc Tokyo Japan) as detailed in the Additional File 11.

Statistical Analysis

Continuous data was summarized using descriptive statistics (mean and standard deviation, or median and interquartile range). Categorical data was presented using N and %. Descriptive statistics were produced using Microsoft Excel® (version 1903). GraphPad® Prism was used for the construction of all figures.

Results

Pre-clinical experiment

The feasibility of extracting Plasmodium parasites from blood using apheresis was initially assessed using cultured P. falciparum parasites. The 1% HCT layer contained the greatest concentration of all parasites as determined by 18S qPCR, with a 1.3-fold concentration of all parasites and a 3.7 and 8-fold concentration for female and male gametocytes, compared to pre-apheresis (Additional File 6 Fig. 3 and Tables 1 to 3). The 2% HCT layer contained the greatest concentration of asexual parasites by microscopy (2.7-fold concentration; Additional File 6 Fig. 4A and Table 4). The highest concentration of gametocytes detected by microscopy was seen in the 1% HCT layer (76-fold ; Additional File 6 Fig. 4A and Table 4). Both asexual parasites and gametocytes were also visualised by microscopy in the 1%, 3% and 5% HCT layers (Additional File 6 Fig. 4B and Table 5). These results demonstrated the technical feasibility of the approach and the experiment was allowed to proceed to the clinical stage.

Clinical experiment

The course of P. vivax infection (Fig. 1) followed the same course as demonstrated in previous studies(9). Subjects 1 and 2 were treated with artemether/lumefantrine 10 days post malaria inoculation. To augment pre-apheresis parasitemia, treatment of subjects 3 and 4 was delayed to day 11. Apheresis could be delayed safely as the clinical signs observed for subjects 3 and 4 were mild enough for the artemether/lumefantrine treatment, only administered after apheresis, to be postponed by 24 hours. All subjects became 18S qPCR negative for parasites within 72 hours of treatment initiation.

A summary of the key differences in the planning of the clinical trial in each of the four subject cohorts is shown in Table 1. During cohort 4 a red cell depletion was carried out, producing an intermediate bag sample, followed by a second apheresis procedure on the red cell depletion product. The second apheresis procedure involved sampling of ~ 100 ml of the lowest HCT layers of the sample (final bag) followed by 100mls of the subsequent lowest HCT layers (spare bag) and then the remainder (waste bag) (Additional File 6 Fig. 2). A schematic of the sampling that occurred during cohort 4 can be seen in Additional File 6 Fig. 2.

Parasitemia as measured by 18S qPCR in all four subjects. Day 0 represents the day of inoculation. Apheresis occurred on day 10 for subjects 1 and 2, and day 11 for subjects 3 and 4.

Safety

A total of 68 AEs occurred in the 4 subjects (Table 2). No Serious AEs were reported. The majority of AEs were mild or moderate. Five severe AEs occurred in 4 subjects: one episode of neutropenia (0.68×10^9/L [$0.45 \times$ LLN]; duration 8 days), two of lymphopenia (0.43×10^9/L [$0.43 \times$ LLN] and 0.33×10^9/L [$0.33 \times$ LLN]; both lasting 3 days), and two of fever (both 40.2 °C; duration 30 minutes and 25 minutes). All severe AEs were transient and resolved by the end of the study. The majority of AEs (54/68; 79.4%) were attributed to malaria, while 9/68 (13.2%) were attributed to apheresis. These included neutropenia (one subject nadir 0.68×10^9/L) which was recorded three times due to changes in severity, two cases of lymphopenia (nadir 0.33×10^9/L and 0.75×10^9/L) and two cases of leukopenia (nadir 2.2×10^9/L and 2.4×10^9/L). One subject had an episode of herpes labialis (herpes simplex virus-1 PCR positive). One subject experienced mild hypophosphataemia (0.70 mmol/L) of two days duration. All AEs attributed to apheresis apart from the case of hypophosphataemia were considered to be possibly related to apheresis, malaria or a combination of both.

Table 2. Summary of the main safety findings

	Subject 1	Subject 2	Subject 3	Subject 4	Total AEs
s	0	0	0	0	0
	20	13	15	20	68
related to malaria	13	8	14	19	54
related to apheresis	2	3	0	4	9
: temp °C	40.2	38.8	40.2	39.6	N/A
: malaria clinical score	8	1	2	7	N/A
profen use	400 mg × 5	400 mg × 5	400 mg × 5	nil	N/A
taminophen use	1 g × 4; 500 mg × 1	1 g × 2	1 g × 4	500 mg × 2; 1 g × 2	N/A
k ALT (IU/L)	111	118	47	80	N/A
k AST (IU/L)	83	57	42	44	N/A
elet Nadir (× 10^9/L)	119	98	99	75	N/A
imum drop in haemoglobin from baseline (g/L)	17	9	25	20	N/A
phocyte Nadir (× 10^9/L)	0.33	0.43	0.58	0.75	N/A
k parasitemia (parasites/mL)	15943	35156	64243	44431	N/A

SAE; serious adverse event, AE; adverse event. Summary of the main safety findings encountered during the study.

Characteristics of samples collected by apheresis

The red blood cell counts of the various HCT layers were generally in alignment with what would be expected (Supplementary Table 6), except in two subjects. In subject 2 the red blood cell counts of the 2% HCT layer was closer to what would be expected from a 3% HCT layer and vice versa, and the 8% HCT sample in subject 3 had a HCT of 11%. The cell composition of samples collected using apheresis in subjects 1 to 3 (Supplementary Fig. 5A) showed an ~ 60 to 170-fold decrease in the RBC:WBC ratio from pre-apheresis samples compared to apheresis samples. Among samples collected by apheresis from subject 4, where a double apheresis process was undertaken, the RBC:WBC ratio was close to that of the pre-apheresis sample (Supplementary Fig. 5B), with the exception of the final bag sample (3% HCT). Reticulocyte counts measured on the Sysmex analyser were the highest in subject 2: 0.23 × 10^9 /L (reference range for whole blood: 25–120 × 10^9/L).

Concentration of asexual parasites

18S qPCR targets the highly conserved plasmodium 18S ribosomal RNA gene present in asexual parasites and gametocytes [14, 18]. However, based on the P. vivax life cycle, microscopy and P. vivax female gametocyte qRT PCR data (pvs25) it was determined that the vast majority of parasites, detected using 18S qPCR, were asexual parasites.
No single HCT layer contained > 40% of all the recovered P. vivax asexual parasites (Fig. 2). An increase in parasite concentration occurred as HCT increased in subjects 1 to 3 (Fig. 2A), with some variation in relative enrichment of parasites in apheresis samples compared to pre-apheresis samples at any given HCT (Fig. 2A). The highest concentration achieved was a 4.9-fold increase in parasite density in the 7% HCT layer in subject 1 (Fig. 2A and Additional File 6 Table 7). There was no apparent enrichment of parasites when the procedure was modified to include a second apheresis process (subject 4; Fig. 2B).

Fold enrichment of parasites as determined by 18 qPCR in samples collected using apheresis compared to the pre-apheresis samples in subjects 1 to 3 (A) and subject 4 (B).

When parasite concentration was adjusted for RBC count, all apheresis samples collected from subjects 1 to 3 demonstrated enrichment for asexual parasites compared to pre-apheresis (Fig. 3A). In general, when parasite counts were corrected for RBC count, parasite enrichment was highest in the low HCT samples with the highest enrichment detected in the 0.5% HCT sample in subject 3 (Fig. 3A and Additional File 6 Table 7; 138-fold compared to pre-apheresis). The relative concentration of parasites from subject 4, where the second apheresis procedure was performed, was observed in the lowest HCT samples. In particular, the final bag (3% HCT) and the spare bag (5% HCT) samples had relative enrichment levels of 20- and 8-fold respectively compared to pre-apheresis (Fig. 3B and Additional File 6 Table 7).

Fold enrichment of parasites corrected for red blood cell counts as determined by 18S qPCR in samples collected using apheresis compared to the pre-apheresis sample in subjects 1, 2 and 3 (A) and subject 4 (B).

Concentration of P. vivax gametocytes

Analysis of the apheresis samples from subject 4, where the double apheresis process was undertaken, demonstrated an increase in the level of female gametocytes of 1.45-fold compared to pre-apheresis, as determined by levels of the gametocyte-specific transcript pvs25 qRT PCR (Fig. 4B and Additional File 6 Table 8).

Subjects 1, 2 and 3 demonstrated a reduction in the level of female gametocytes compared to pre-apheresis (Fig. 4A and Additional File 6 Table 8). In most cases the reduction was > 10-fold. Percoll concentration of whole blood taken pre-apheresis resulted in a significant enrichment (up to 45-fold in subject 3) of pvs25 compared to pre-apheresis samples not enriched with percoll (Fig. 4C and Additional File 6 Table 8).

Fold enrichment of female P. vivax gametocytes determined by pvs25/ml in samples collected using apheresis compared to the pre-apheresis sample in subjects 1, 2 and 3 (A) and subject 4 (B). Fold enrichment of female P. vivax gametocytes determined by pvs25/ml in samples collected using apheresis compared to the pre-apheresis sample and pre-apheresis samples enriched with percoll in subjects 1, 2 and 3 (C).

In subject 1 the percoll enrichment experiment failed due to technical issues resulting from suboptimal processing of the sample.

When gametocyte concentrations were corrected for red blood cell counts, enrichment levels were generally higher in the lower HCT samples (Fig. 5A and 5B; Additional File 6 Table 8). Relative enrichment of gametocytes in subjects 1, 2 and 3 was lower than for total parasites, with a maximum enrichment of 6.2-fold in the 0.5% HCT layer (Additional File 6 Table 8). In subjects 3 and 4 enrichment relative to red blood cell count was observed (Fig. 5A and 5B). In subject 4, the greatest enrichment of gametocyte transcripts in the double apheresis process was observed in the spare bag (5% HCT) with an enrichment of 6.1-fold (Fig. 5B and Additional File 6 Table 8).

Fold enrichment of female P. vivax gametocytes corrected for RBC counts determined by pvS25 collected using apheresis compared to the pre-apheresis sample in Subjects 1, 2 and 3 (A) and subject 4 (B).

Flow Cytometry

In subjects 2 and 3, it was observed that as the haematocrit decreased there was an increase in the percentage of WBCs (Fig. 6A and 6B). A small percentage of SYBR Green I + cells and CD45-, which could represent either parasitised RBCs or reticulocytes, were detected in samples from subjects 2 and 3, where 2–3% HCT pooled samples from subject 3 had the highest concentration (0.59%) of SYBR Green I + and CD45- (Fig. 6B). In subject 4, where CD71 antibody staining was used to identify reticulocytes, the highest levels of reticulocytes (CD71 + cells) were observed in the final bag (3% HCT) and the spare bag (5% HCT) (Fig. 6C). Microscopic analysis of these samples suggested that the reticulocyte population detected by flow cytometry consisted of uninfected reticulocytes. No parasitised RBCs or reticulocytes could be detected by flow cytometry.

Percentage of CD45-/SYBR + in samples obtained from subject 2 (A) and subject 3 (B). The percentage of (CD71+) reticulocytes from subject 4 (C).

Microscopy

The high concentration of WBCs in apheresis samples, meant many of the thick and thin blood smears were extremely difficult to read (Additional File 6 Tables 9 and 10).

Among samples where it was possible to read the films, parasite counts were low, and in alignment with the counts obtained by qPCR (Additional File 6 Tables 9 and 10). Notwithstanding the technical difficulties in reading the slides and the low parasite counts, no apparent concentration of parasites was observed in any of the apheresis samples compared to pre-apheresis. The 3% HCT sample in subject 3 contained 7 parasitised cells compared to 0 in the pre-apheresis sample and the 1% HCT sample in subject 1 demonstrated a 4-fold increase in the number of visualised parasites compared to pre-apheresis. In general, higher parasite numbers were seen in the lower HCT layers. The vast majority of parasitized RBCs contained ring form parasites, with trophozoites and gametocytes observed in samples from subjects 3 and 4 only (Additional File 6 Tables 9 and 10).

Mosquito transmission

Membrane feeding of pre-apheresis or post percoll enrichment samples were undertaken in all 4 subjects, but for logistical reasons membrane feeding on apheresis samples could only be undertaken in subjects 1 and 4 (Table 3).

None of the samples from subject 1 or 2 resulted in successful mosquito transmission (Table 3). In subject 3, the infection rate from the percoll-enriched sample was 5.8-fold higher than the infection rate from the non-enriched pre-apheresis sample (99% vs 17.2% [Table 3]). Likewise, in subject 4, the infection rate from the final bag (3% HCT) was 5.5-fold higher than the infection rate from either the pre-apheresis sample or the intermediate bag (64%HCT) (pre-apheresis: 3.6%, intermediate bag: 4%, final bag: 20% [Table 3]).

The final sample bag (3% HCT) in subject 4 was the only sample obtained using apheresis that demonstrated an increase in transmission over samples obtained pre-apheresis. Due to logistic issues the spare bag (5% HCT), which contained the greatest enrichment for gametocytes compared to pre-apheresis when adjusted for red cell counts in subject 4 (Fig. 5B) was not subject to membrane feeding.

Table 3. Mosquito infection rates following membrane feeding assays.

le	Subject 1				Subject 2	Subject 3		Subject 4			
	Pre-apheresis (with-Percoll enrichment)	Apheresis samples			Pre-apheresis (with-Percoll enrichment)	Pre-apheresis (without-Percoll enrichment)	Pre-apheresis (with-Percoll enrichment)	*Pre-apheresis (without-Percoll enrichment)	Apheresis samples		
		1% HCT	2% HCT	3% HCT					Intermediate (64% HCT)	Final (3% HCT)	Waste (42% HCT)
ate ; otal ;	127/133 [95.5%]	101/103 [98.1%]	103/104 [99%]	114/116 [98.3%]	75/77 [97.4%]	102/103 [99%]	107/107 [100%]	29/29 [100%]	28/29 [97%]	26/26 [100%]	30/30 [100%]
rate . ;/No. ;	23/133 [17.3%]	3/103 [2.9%]	6/104 [5.8%]	11/116 [9.5%]	7/77 [9.1%]	6/103 [5.8%]	5/107 [4.7%]	1/29 [3.4%]	3/29 [10.3%]	1/26 [3.8%]	1/30 [3.3%]
rate es No. ;	0/110[0%]	0/105 [0%]	0/98 [0%]	0/105 [0%]	0/77 [0%]	16/93 [17.2%]	99/100 [99%]	1/28 [3.6%]	1/25 [4%]	5/25 [20%]	0/24 [0%]
])											

Only subjects 1 and 4 involved the testing of samples collected using apheresis. For logistic reasons, MFA was not carried out in subject 4. Following consideration of gametocyte levels, DMFA was not carried out in subject 2. Reported parameters include feeding rate of mosquitoes, adult mosquito mortality rate after feeding and mosquito infection rate.

*MFA without percoll enrichment to save on blood volume draw for safety reasons.

Additional 18S qPCR testing

To investigate for possible accumulation of parasites in the magnets and tubing structures in the apheresis cassette, qPCR testing was carried out on three blood clots with diameters of up to 1 cm found near two magnets, and a silo like structure that formed part of the single use apheresis tubing and processing cassette from subject 3 (Additional File 6 Fig. 11). Clots were thoroughly homogenised and tested by 18S qPCR. The greatest enrichment observed relative to pre-apheresis whole blood was 1.4-fold in the apheresis cassette magnet 2 sample (Additional File 6 Fig. 12 and Table 11).

To investigate whether haemolysis may be taking place in the apheresed blood resulting in release of parasite DNA into the extracellular fluid, plasma from apheresis samples in subject 3 was collected by centrifugation of the sample, with the plasma subject to 18S qPCR testing. However, no significant accumulation of parasite DNA was detected in the plasma compared to pre-apheresis (Additional File 6 Fig. 12 and Table 11).

Discussion

Using apheresis it was possible to achieve modest concentration of both asexual and gametocyte stages of P. vivax. However, the modest level of parasite enrichment (4.9-fold and 1.45-fold per ml of sample for asexual parasites and gametocytes respectively) was insufficient to meet the objectives for the collection of parasites for downstream research.

Results of this study suggest that apheresis in healthy subjects infected with blood-stage P. vivax parasites is safe. No serious adverse events were encountered, with all adverse events having resolved by the end of study. The majority of adverse events were malaria related, and in line with previous P. vivax IBSM studies(7, 8). Adverse events related to apheresis consisted largely of asymptomatic transient reductions in haematology parameters.

After correction for red blood cell number, parasite quantitation by qPCR suggested that both asexual parasitemia and gametocytes were preferentially concentrated in the lower HCT layers (Figs. 3 and 5) which was consistent with the previously published observations of the buoyancy of Plasmodium parasites(24).

Several scenarios were considered to explain why concentration of parasites using apheresis was lower than expected. Firstly, as gametocyte concentrations in apheresis samples were around the level of detection of the pvs25 by qRT-PCR, minor variation in concentration may have been difficult to quantify(25). Secondly, infected

RBCs containing magnetic hemozoin(26) may have attached to ferromagnetic components of the apheresis apparatus. Thirdly, it is possible that lysis of asexual parasites and gametocytes occurred during the apheresis procedure. This latter hypothesis is supported by a recent report suggesting that parasite maturation results in increasing fragility of P. vivax infected red blood cells(27). Although it may have been possible to assess for low level haemolysis during the procedure, for example by measuring haptoglobin levels, controlling for a range of other variables would have been difficult.

An enhanced level of transmission to mosquitoes compared to whole blood samples collected pre-apheresis was only observed on one occasion (final sample bag [3% HCT] in subject 4), corresponding to the higher gametocyte concentration in this sample compared to the pre-apheresis whole blood (Fig. 6B). A possible explanation of the low success in the transmission studies was the difficulty in maintaining tight temperature control to prevent exflagellation of male gametocytes(28), thereby negatively impacting gametocyte infectivity(29). Blood was most vulnerable to a temperature drop whilst in the apheresis equipment itself. It was not possible to heat apheresis equipment, and it was deemed impractical to heat the room where apheresis took place to > 35° C. Temperature monitoring was not possible during the experiments. However, the demonstration of transmission success in the final bag (3% HCT) indicates that at least some gametocytes were maintained within a temperature range that did not trigger exflagellation. Regardless of the underlying cause, recent reports of success in improving concentration of gametocytes and enhanced transmission by either percoll(9, 10) or magnetic bead(30) enrichment suggests that such methods are superior for concentration of gametocytes for mosquito transmission experiments.

Each of the vials from the HMP bank used in this study to infect subjects contain 2.08×10^6 parasites. Based on the greatest level of asexual enrichment per ml of sample observed (7% HCT, subject 1), calculations suggest that apheresis alone can create parasites vials with a maximum of 7.92×10^4 parasites (Additional File 6). Therefore, to create a HMP bank using an apheresis approach equivalent to the one used to infect volunteers in this study a > 25-fold increase in pre-apheresis parasitemia would be required, equating to > 650,000 parasites/ml. Attaining such high parasitemias would likely be associated with a greater number of AEs and significant ethical concerns. Therefore, unless significant improvements in enrichment can be attained, apheresis should not be used to create HMP banks, and the current practice of collecting blood by venesection is preferable.

Strengths of the study include the wide sampling across HCT layers (1%, 2%, 3%, 5% and 7%) and the use of multiple methods to enumerate parasites (flow cytometry, microscopy and qPCR). Although only a small number of subjects were studied using this approach, the lack of promising data meant that continuation of the trial was deemed inappropriate by the safety review team and the study was terminated.

Conclusion

Given the moderate levels of enrichment and the significant ethical, financial and logistical concerns surrounding *P. vivax* IBSM studies, we consider that further apheresis studies are not warranted at this point. If apheresis were to be used again in the context of Malaria VIS, it should ideally only be carried out in subjects with normal haematological parameters at screening to avoid potential complications or the need for blood transfusion.

Abbreviations

volunteer infection studies (VIS), controlled human malaria infection (CHMI) studies, induced blood stage malaria (IBSM), red cell exchange (RCE), Queensland Institute of Medical Research (QIMR), Royal Brisbane and Women's Hospital (RBWH), Human Research Ethics Committee (HREC), Good Clinical Practice (GCP), haematocrit (HCT), common terminology of clinical trial adverse events (CTCAE), continuous mononuclear cell collection (CMNC), polymerase chain reaction (PCR), quantitative polymerase chain reaction (qPCR), quantitative reverse transcriptase polymerase chain reaction (qRT PCR), white blood cells (WBCs), red blood cells (RBCs), membrane feeding assays (MFA), direct membrane feeding assays (DMFA), polymorphonuclear (PMN), lower limit of normal (LLN), upper limit of normal (ULN), serious adverse event (SAE), adverse event (AE).

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the protocol approved by the QIMR Berghofer Human Research Ethics Committee (HREC) and the RBWH HREC, the principles of the Declaration of Helsinki (recommendations guiding medical doctors in biomedical research involving human subjects, Fortaleza, Brazil 2013), the NHMRC National Statement on Ethical Conduct in Human Research (2007), and the Notes for Guidance on Good Clinical Practice (GCP) (CPMP/ICH/135/95) as adopted by the Australian Therapeutic Goods Administration (2000). All amendments and addenda to the protocol were similarly submitted to the QIMR Berghofer HREC and the RBWH HREC for approval prior to their implementation. All subjects gave written informed consent before being included in the study.

Consent for publication

All subjects gave written informed consent for their anonymized data to be published.

Availability of data and materials

Data collected for this study will be made available immediately after article publication with no end date. De-identified datasets containing the variables analysed for the primary and secondary objectives will be made available as well as other supporting documents (e.g., protocol and informed consent). Investigators who seek access to individual subject data will contact the corresponding author (AO) to receive instructions on the formal request process, which will include the submission of a brief proposal. The proposal will be reviewed for merit and feasibility by the corresponding author (AO). Investigators will be notified of the decision within 30 days of receipt. If the request is accepted, a data transfer agreement covering relevant conditions will be required.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AO oversaw study conception, design of the study, study conduct, data collection, data analysis and manuscript production. KM oversaw the apheresis procedure for each subject and involved in study conception, design of the study, study conduct, data collection, data analysis and approval of the manuscript. GL provided clinical oversight for the apheresis procedure for each study subject and was involved in study conception, design of the study, data analysis and approval of the manuscript. REW was involved in study conception, design of the study including the production of the study protocol and patient information and consent form. EM was involved in design of the study, study conduct, data collection, data analysis and approval of the manuscript. HM was involved in design of the study, study conduct, data collection, data analysis and approval of the manuscript. JG was involved in design of the study, study conduct, data collection, data analysis and approval of the manuscript. MR was involved in design of the study, study conduct, data collection, data analysis and approval of the manuscript. ZPI was involved in design of the study, study conduct, data collection, data analysis and approval of the manuscript. RP was involved in design of the study, study conduct, data collection, data analysis. SW was involved study conduct, data collection and approval of the manuscript. DGL was involved in design of the study, data analysis and approval of the manuscript. GR study conduct and data collection. KAC was involved in design of the study, study conduct, data collection, data analysis and approval of the manuscript. FA was involved in design of the study, study conduct, data collection, data analysis and approval of the manuscript. JMc provided senior scientific input and clinical oversight on study conception, design of the study, study conduct, data analysis and manuscript production.

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Additional Material

Additional_file_1_Apheresis_of_subjects_with_induced_blood_stage_p._vivax_protocol.docx

Additional_file_2_Apheresis_Cohort_1_Standard_Operating_Procedure.docx

Additional_file_3_Apheresis_Cohort_2_Standard_Operating_Procedure.docx

Additional_file_4_Apheresis_Cohort_3_Standard_Operating_Procedure.docx

Additional_file_5_Apheresis_Cohort_4_Standard_Operating_Procedure.docx

Additional_file_6_Supplementary_Material.docx

Additional_file_7_Apheresis_Cohort_1_Laboratory_Standard_Operating_Procedure.docx

Additional_file_8_Apheresis_Cohort_2_Laboratory_Standard_Operating_Procedure.docx

Additional_file_9_Apheresis_Cohort_3_Laboratory_Standard_Operating_Procedure.docx

Additional_file_10_Apheresis_Cohort_4_Laboratory_Standard_Operating_Procedure.docx

Additional_file_11_Spectra_Optia_Brochur_System_Overview.pdf

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Figures

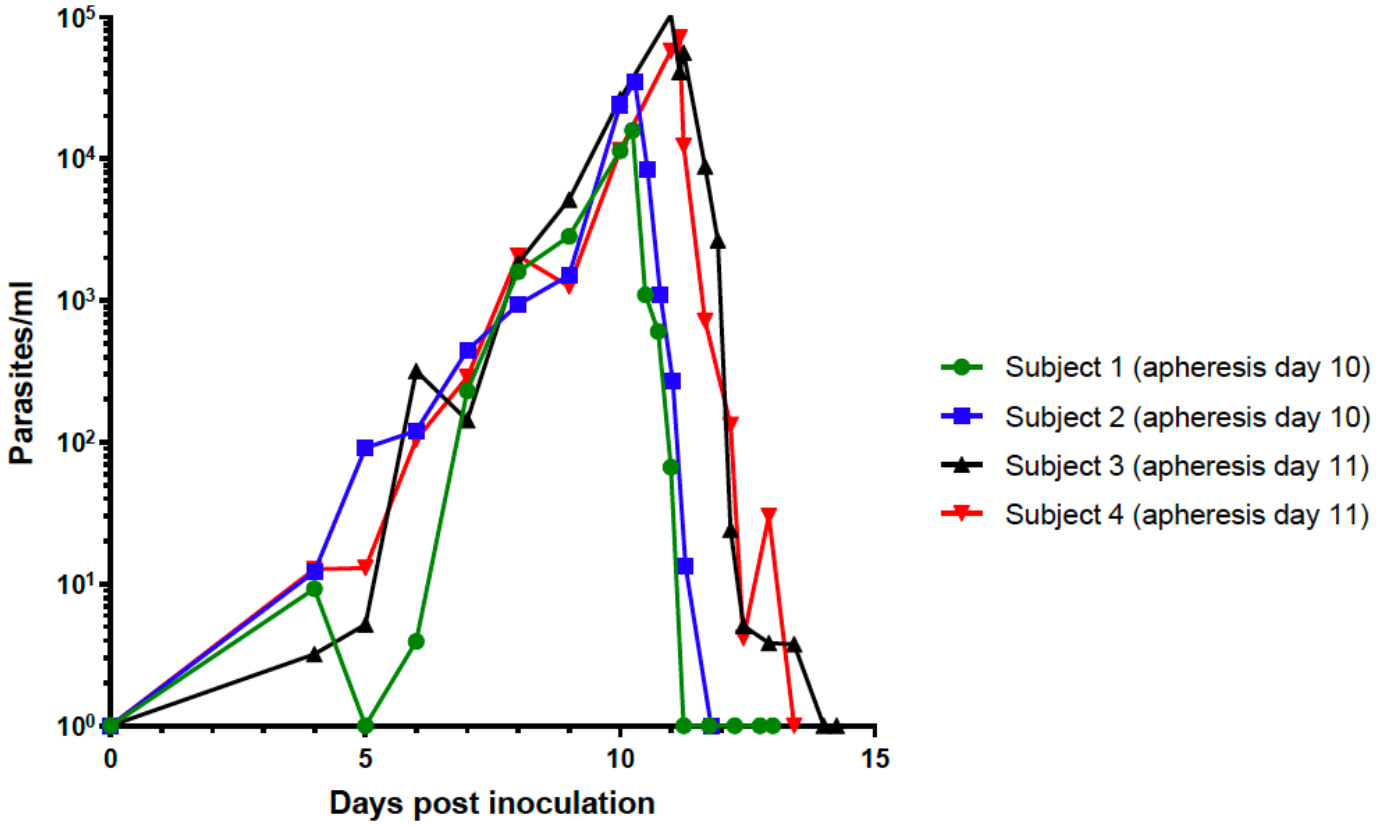

Figure 1

Parasite growth curves

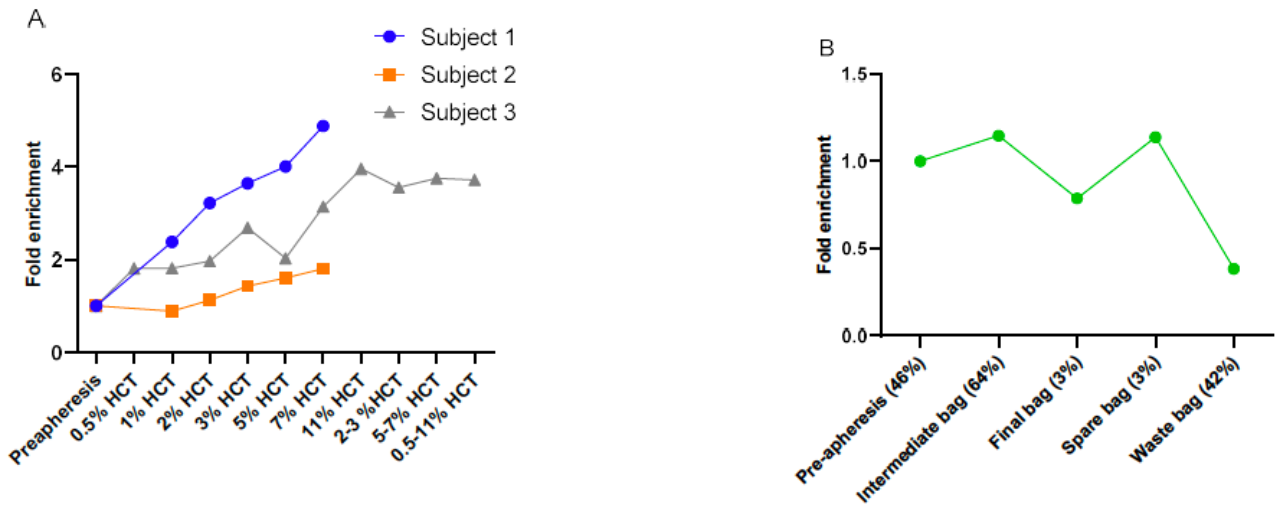

Figure 2

Fold enrichment of parasites/ml determined by 18S qPCR

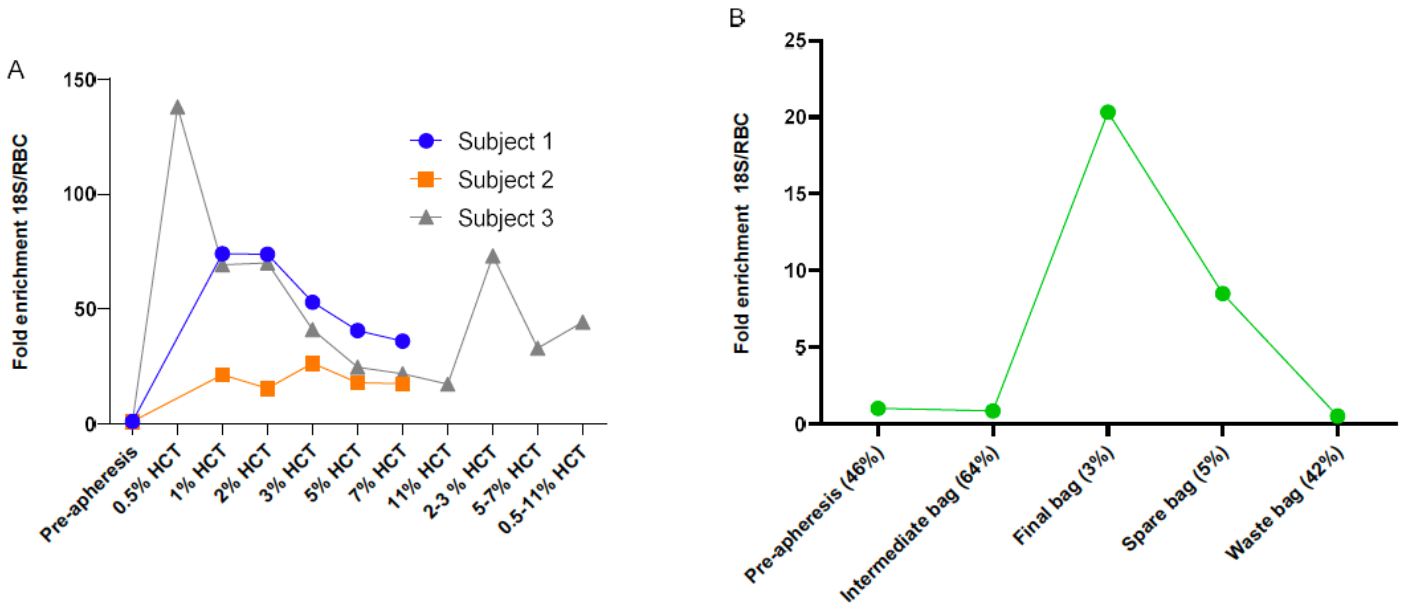

Figure 3

Fold enrichment of parasites/ml determined by 18S qPCR/RBC

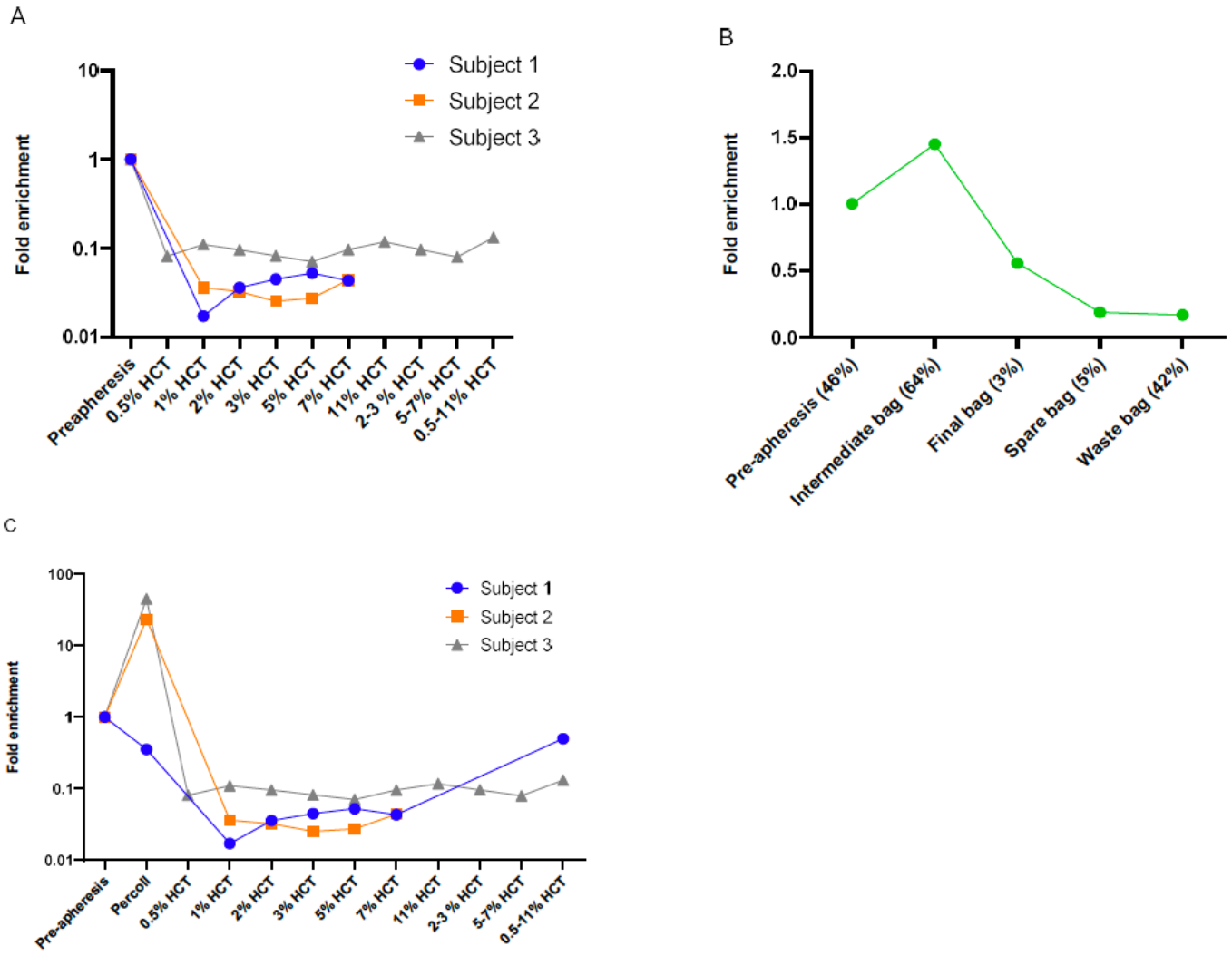

Figure 4

Fold Enrichment female P. vivax gametocytes determined by pvs25/ml

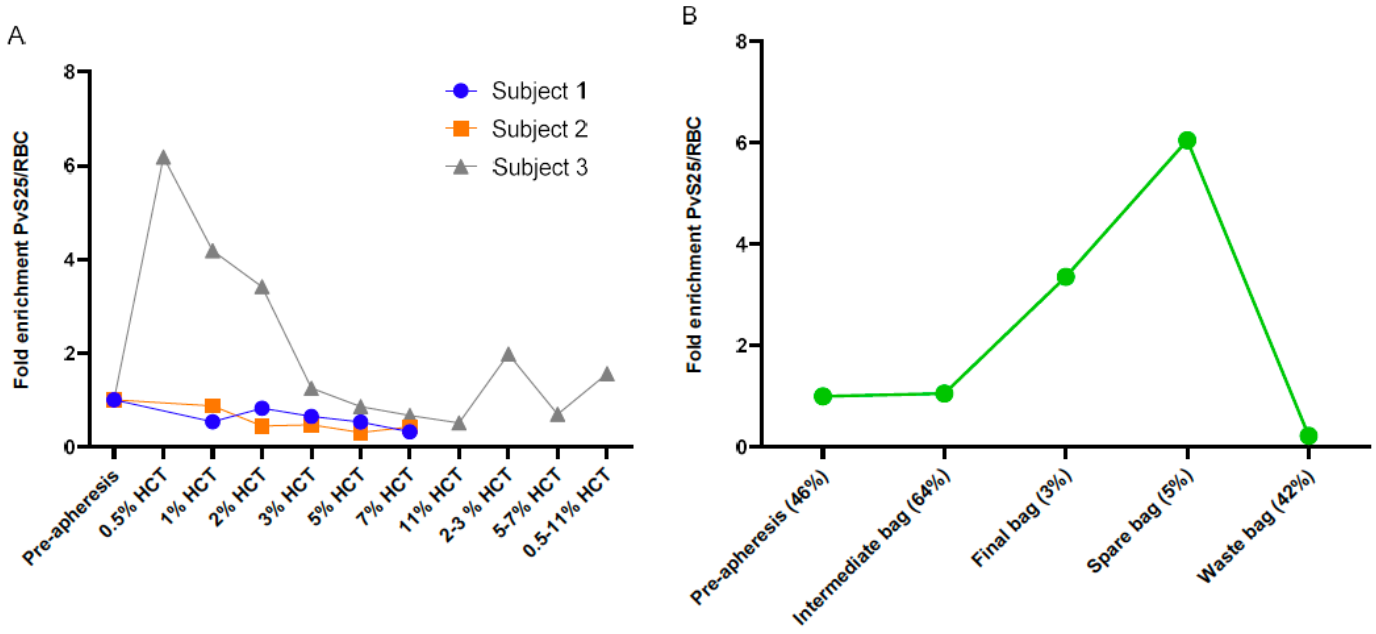

Figure 5

Fold Enrichment female P. vivax gametocytes determined by pvs25/RBC.

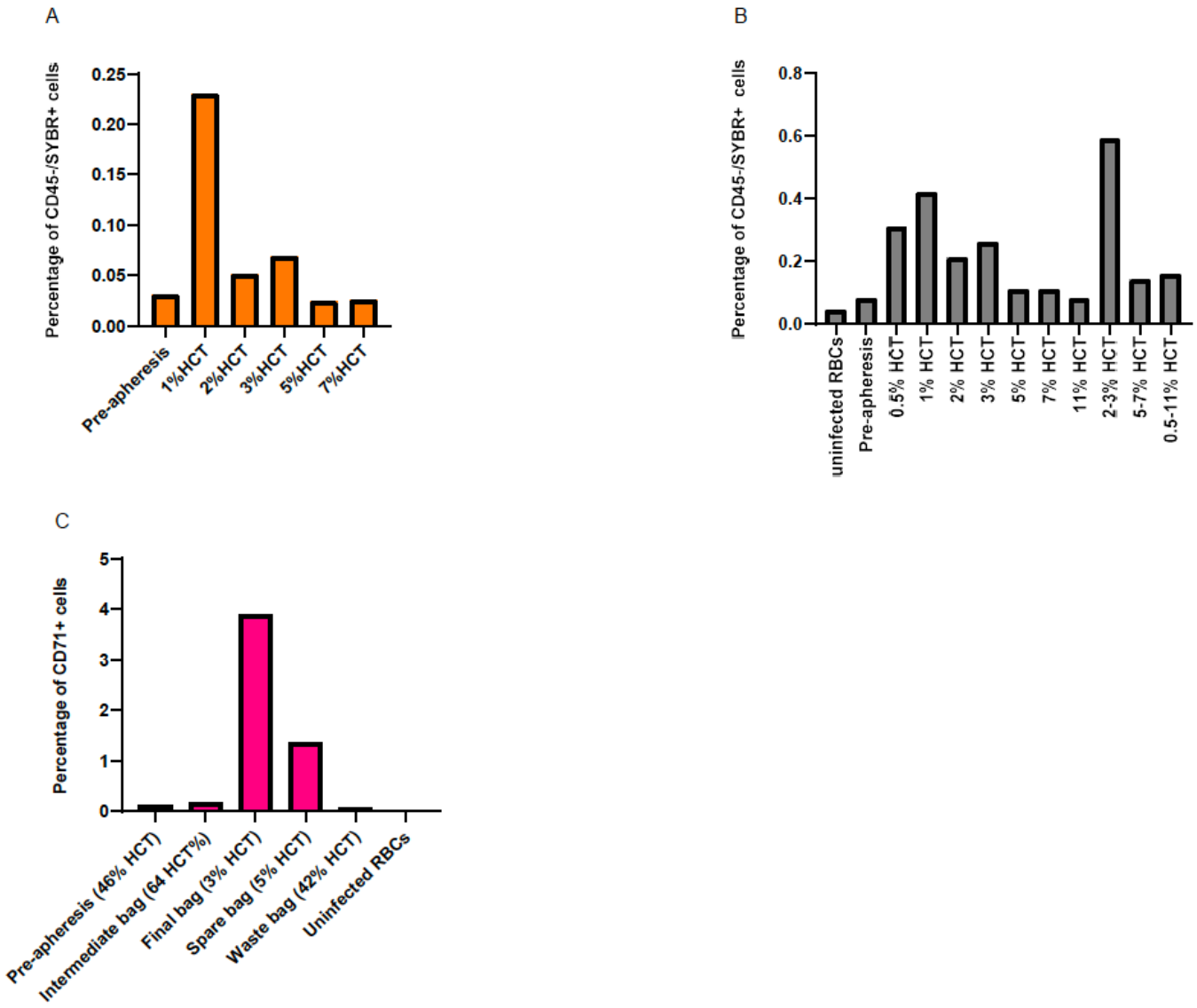

Figure 6

Flow cytometry cohorts 2 to 4

Supplementary Files

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