Purification of *Plasmodium* and *Babesia*- infected erythrocytes using a non-woven fabric filter

Tao, Z.Y.[1,2*], Liu, W.P.[1,2], Dong, J.[3], Feng, X.X.[4], Yao, D.W.[4], Lv, Q.L.[1], Ibrahim, U.[1,2], Dong, J.J.[1], Culleton, R.[5], Gu, W.[1], Su, P.P.[1], Tao, L.[1], Li, J.Y.[1], Fang, Q.[1,2*] and Xia, H.[1,2*]

[1]Department of Microbiology and Parasitology, Bengbu Medical College, 2600# Donghai Avenue, Bengbu 233030, China
[2]Anhui Provincial Key Laboratory of Infection and Immunology, Bengbu Medical College, 2600# Donghai Avenue, Bengbu 233030, China
[3]Department of Microbiology, Bengbu Center for Disease Control and Prevention, 700# Huayuan Road, Bengbu 233000, China
[4]College of Veterinary Medicine, Nanjing Agricultural University, No.1 Weigang Road, Nanjing 210095, China
[5]Department of Molecular Parasitology, Proteo-Science Center, Ehime University, Shitsukawa, Toon, Ehime 791-0295, Japan
*Corresponding author e-mails: Taozhiyong@bbmc.edu.cn (Zhi-Yong Tao); fq333@sohu.com (Qiang Fang); xiahui@ bbmc.edu.cn (Hui Xia)
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Abstract. The purification of parasite-infected erythrocytes from whole blood containing leucocytes is crucial for many downstream genetic and molecular assays in parasitology. Current methodologies to achieve this are often costly and time consuming. Here, we demonstrate the successful application of a cheap and simple Non-Woven Fabric (NWF) filter for the purification of parasitized red blood cells from whole blood. NWF filtration was applied to the malaria-parasitized blood of three strains of mice, and one strain of rat, and to *Babesia gibsoni* parasitized dog blood. Before and after filtration, the white blood cell (WBC) removal rates and red blood cell (RBC) recovery rates were measured. After NWF filter treatment of rodent malaria-infected blood, the WBC removal rates and RBC recovery rates were, for Kunming mice: 99.51%±0.30% and 86.12%±8.37%; for BALB/C mice: 99.61%±0.15% and 80.74%±7.11%; for C57 mice: 99.71%±0.12% and 84.87%±3.83%; for Sprague-Dawley rats: 99.93%±0.03% and 83.30%±2.96%. Microscopy showed WBCs were efficiently removed from infected dog blood samples, and there was no obvious morphological change of *B. gibsoni* parasites. NWF filters efficiently remove leukocytes from malaria parasite-infected mouse and rat blood, and are also suitable for filtration of *B. gibsoni*-infected dog blood.

INTRODUCTION

Intraerythrocytic parasites such as *Plasmodium* and *Babesia*, are important pathogens of humans and animals. Malaria is a serious public health threat to humans; there are about 219 million cases a year globally (Winzeler *et al.*, 2019). The disease is caused by the intraerythrocytic protozoan parasite *Plasmodium.* Babesiosis is a tick-borne zoonotic protozoan parasitic disease, the symptoms of which are similar to malaria

(Moritz *et al.*, 2016). *Babesia* mainly threatens livestock and companion animals.

Rodent malaria parasites are commonly used in laboratory-based studies of the disease (Craig *et al.*, 2012). There are four species of rodent malaria parasites are used in laboratories worldwide; *Plasmodium chabaudi*, *Plasmodium vinckei*, *Plasmodium yoelii* and *Plasmodium berghei* (Huang *et al.*, 2015) and these are maintained in a large variety of mouse and rat strains. Rodent malaria parasites have informed studies on

malaria pathology, cerebral malaria, antimalarial chemotherapy, malaria immunology, and vaccine development (Zuzarte-Luis *et al.*, 2014; Moussa *et al.*, 2018; Sala *et al.*, 2018).

Babesia gibsoni is endemic in dogs in Asia, Africa and occasionally North America and Europe. It is typically transmitted by tick bite but also by direct blood contamination during, for example, fighting (Cannon *et al.*, 2016). Experimental passage of *B. gibsoni* to beagles is common in laboratory studies on babesiosis.

Separating *Plasmodium* parasite infected red blood cells (iRBC) from leucocytes in infected animal blood is often an essential procedure during laboratory investigations of *Plasmodium* biology, immunology, molecular biology and genomics (Grech *et al.*, 2002; Lu *et al.*, 2011).

There are multiple methods currently available for the removal of leucocytes from whole blood (Venkatesan *et al.*, 2012; Mehlotra *et al.*, 2017; Mkumbaye *et al.*, 2017). Differential centrifugation and the flushing of blood through cellulose powder columns are commonly used methodologies as they are cheap and straightforward, but have relatively low efficacies and are time-consuming. They also suffer from relatively low iRBC recovery rates (Tao *et al.*, 2011). Plasmodipur™ filter filtration is easy to perform and achieves good results but is relatively expensive, especially for large scale use (Nag *et al.*, 2018). Magnetic separation methods are able to separate and concentrate late trophozoites, schizonts, and gametocytes, but hemozoin-loaded microphages can be recovered along with iRBCs, and separation of ring-stage parasites is not possible (Trang *et al.*, 2004; Bhakdi *et al.*, 2010). An optimized method using magnetic nanoparticles conjugated with patient derived antibodies was shown to separate and concentrate multi-stage parasites together, but it required extensive antibody related preparation (Tangchai-keeree *et al.*, 2013).

We have developed a prototype non-woven fabric filtration device (Tao *et al.*, 2011). It is suitable for the processing of small volumes of malaria-infected human blood, with high efficiency in both WBC removal and RBC recovery, and it has been used successfully in research on the genetics of the human malaria parasites *Plasmodium vivax* and *Plasmodium ovale* (Ansari *et al.*, 2016; Pearson *et al.*, 2016). Previously, it was also shown to be effective in the laboratory, where it was used to remove leucocytes from the blood of BALB/c mice infected with *P. yoelii* (Li *et al.*, 2016). Here, we present the results of experiments testing the effectiveness of NWF filtration for purification of iRBCs from the blood of *P. berghei* and *P. yoelii*-infected BABL/c, C57, and Kunming mice and SD rats, and from dog blood infected with *B. gibsoni*.

MATERIALS AND METHODS

Laboratory animals, Parasites and NWF filters

BABL/c, C57, Kunming mice, and SD rats were purchased from the Experimental Animal Center of Anhui Medical University (Anhui, China) (Shang *et al.*, 2009). All mice were female, 6-8 weeks old, weighed 25-30 g; female SD rats were all 8-10 weeks old, and weighed ~200 g. All animals used in this study were maintained according to Bengbu Medical College laboratory regulations. *Plasmodium yoelii* and *P. berghei* parasites were maintained in mice and cryopreserved in liquid nitrogen. Beagle dogs were purchased from Qing Long Shan Laboratory Center (Nanjing, China). *Babesia gibsoni* was isolated from a pet dog and kept in the College of Veterinary Medicine, Nanjing Agricultural University (Nanjing, China). The NWF filters were purchased from Zhixing Bio, Co. LTD (Anhui, China), the design of filter has been improved to connect to a syringe easily. (Li *et al.*, 2017).

Infection of laboratory animals

After propagation in mice, 1×10^6 iRBCs of *P. berghei* or *P. yoelii* were injected intra-peritoneally to each laboratory mouse in 100 µL saline (1×10^7 iRBCs were injected in the case of rat infection, each group con-

tained five animals). Parasitemia was recorded every other day from three days post-infection. Five mL of blood was taken from the *B. gibsoni*-infected beagle dog, and injected into a healthy dog *via* the cephalic vein. Blood smears were prepared and read every five days.

Filtration of malaria-infected animal blood using NWF filters
Infected mice and rats were anesthetized, and 1 mL blood samples were collected using a heparin anticoagulation tube. RPMI 1640 medium (Gibico, USA) was used to dilute each blood sample to 5 mL. The suspension was aspirated into a 10 mL syringe, and pushed through a NWF filter. The process was finished within 1 minute. 0.5 mL cell suspension was collected before and after treatment for WBC, RBC, and genetic tests. Five mL of blood was drawn from the infected beagle dog and diluted to 10 mL with RPMI 1640 and filtered as above. The morphology of *Plasmodium* and *Babesia* parasites were examined by microscopy with Giemsa's solution.

WBC removal and RBC recovery rates
WBC counts were measured by Nageotte chamber microscopy (Brand, Wertheim, Germany) (Petersson *et al.*, 2017). WBC removal rate = (1-WBC after filtration/WBC before filtration) ×100%; RBC was determined by automated blood cell counter (Mindray, Shenzhen, China), RBC recovery rate = RBC after filtration/RBC before filtration×100%.

Detection of mouse DNA in NWF filter treated mouse blood
A pair of primers (Forward: 5'-AGGCCGG TGCTGAGTATGTC-3', Reverse: 5'-TGCCT GCTTCACCACCTTCTTG-3') for detecting the GAPDH gene were synthesized by Sangon Biotech (Shanghai, China). Genomic DNA was extracted by Axyprep Multisource Genomic DNA Miniprep Kit (Axygen, Suzhou, China) from blood samples of *P. berghei*-infected BALB/c mice before and after filtration. The DNA concentrations of the two samples were adjusted to the same concentrations (10 ng/µL), then were serially diluted 10-fold from 10 ng/µL to 1×10^{-4} ng/µL. Ex Taq kit (Takara Bio, Shiga, Japan) was used for PCR, and the conditions were 95°C 5 min, 30 cycles of 95°C 15 s, 56°C 30 s, 72°C 45 s, and a final extension at 72°C for 5 min. PCR products were analyzed by 1.5% agarose gel.

Infection of BALB/c mice with NWF filtered *P. berghei*-infected blood
P. berghei-infected mouse blood was collected and filtered aseptically as described previously, and the blood was diluted by sterile saline solution for mouse infection. Five BALB/c mice were injected intraperitoneally with 100 µL RBC suspension (1×10^6 parasites) respectively. After infection, blood samples were collected at days 4, 5, 6, and 7 to examine the parasitemia.

Ethics statement
All animal experiments were approved by the Experimental Animal Management and Ethics Committee of Bengbu Medical College, Bengbu, China (BBMC2017-074).

Statistical analysis
The WBC removal rates, RBC recovery rates and 95% confidence intervals (CI) were calculated using Microsoft Excel 2003.

RESULTS

Morphological characterization of parasites following NWF filtration
After filtration with the NWF filters, the *P. berghei* and *P. yoelii*-infected mouse blood samples and *B. gibsoni*-infected dog blood samples were examined by microscopy with Giemsa's solution stain both in thin and thick blood films. There were no morphological changes in any parasite species observed by thin-film microscopy. There was a large reduction in the numbers of WBCs observed by microscopy in both mouse and dog blood following NWF filter treatment by thick-film microscopy (Figure 1).
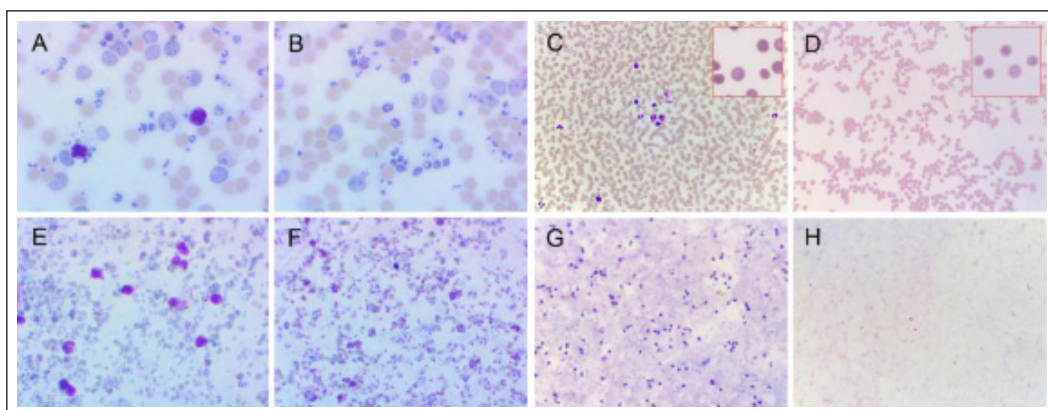
Figure 1. Microscopy of infected blood samples before and after filtration with the NWF filter.
(A) *P. berghei*-infected BALB/c mouse blood, thin film, before filtration (1000×); (B) *P. berghei*-infected mouse blood, thin film, after filtration (1000×); (C) *B. gibsoni*-infected canine blood, thin film, before filtration (400×, 1000× in red rectangle); (D) *B. gibsoni*-infected canine blood, thin film, after filtration (400×, 1000× in red rectangle); (E) *P. berghei*-infected blood, thick film, before filtration (1000×); (F) *P. berghei*-infected blood, thick film, after filtration (1000×); (G) *B. gibsoni*-infected canine blood, thick film, before filtration (400×); (H) *B. gibsoni*-infected dog blood, thick film, after filtration (400×).

Table 1. WBC removal and RBC recovery following NWF filtration for *P. berghei*-infected rodent blood samples

Animals	n	WBC removal rates X±SD (95% CI)	RBC recovery rates X±SD (95% CI)
Kunming mice	5	99.51%±0.30% (99.14%–99.87%)	86.12%±8.37% (75.73%–96.52%)
BALB/c mice	5	99.61%±0.15% (99.43%–99.80%)	80.74%±7.11% (71.91%–89.57%)
C57 mice	5	99.71%±0.12% (99.56%–99.85%)	84.87%±3.83% (80.11%–89.63%)
SD rats	5	99.93%±0.03% (99.90%–99.97%)	83.30%±2.96% (79.62%–86.97%)

WBC removal and RBC recovery efficacy using NWF filters
BALB/c, C57, Kunming mice and SD rat groups (n=5 each) were included. After NWF filtration the WBC removal rates were all above 99.5%, and the RBC recovery rates were all above 80.0% (Table 1).

Semi-quantitative measurement of mouse GAPDH gene copy number in whole blood pre and post NWF filtration
For *P. berghei*-infected BALB/c mice, semi-quantitative PCR gel analysis showed that the mouse GAPDH gene copy number in blood decreased about 100 fold following NWF filtration (Figure 2).

Infection of mice with filtered *P. berghei* iRBC
Two groups of BALB/c mice (n=5, each) were infected with $1×10^6$ parasites of filtered or unfiltered *P. berghei* iRBC derived from the same blood sample. All mice became infected, and the parasitemia of two groups were similar between day 4 to day 7 (Figure 3).
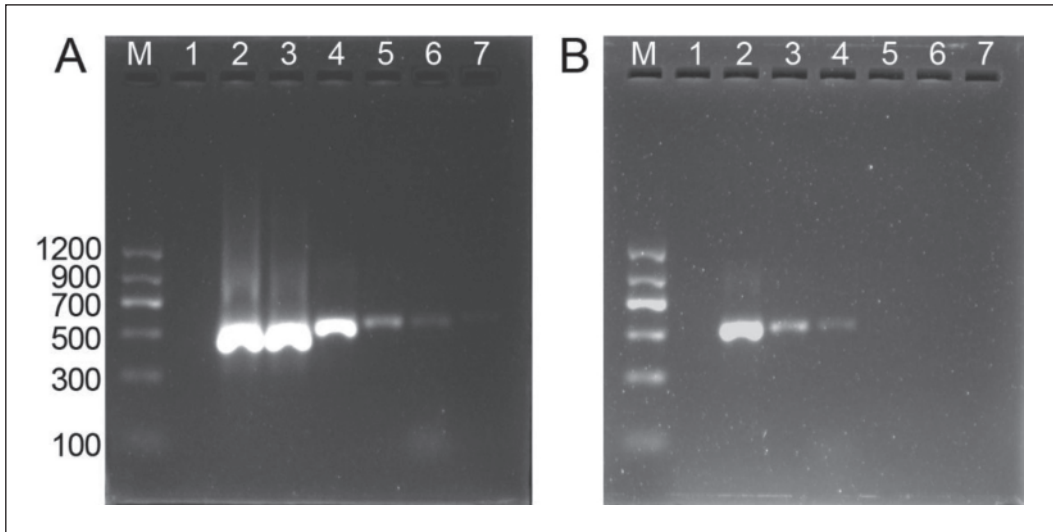
Figure 2. Effect of NWF filtration on GAPDH gene detection determined by PCR.
(A) Before filtration; (B) After filtration; (M) DNA marker; (A1, B1) Negative control; (A2, B2) 10 ng/µL DNA; (A3, B3) 1 ng/µL DNA; (A4, B4) 0.1 ng/µL DNA; (A5, B5) 0.01 ng/µL DNA; (A6, B6) 0.001 ng/µL DNA; (A7, B7) 0.0001 ng/µL DNA.
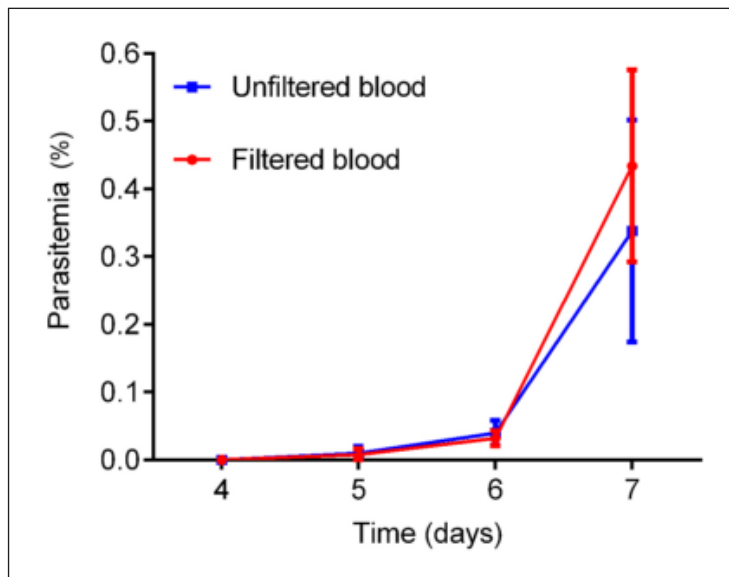

Figure 3. Parasitemia in mice infected with NWF filtered *P. berghei* blood.
The parasitemia of the two groups are not significantly different (P>0.05).

DISCUSSION

Removal of WBCs from intraerythrocytic parasite-infected blood samples is a crucial step in many laboratory studies on intra-erythrocytic parasites (Auburn *et al.*, 2011; Roobsoong *et al.*, 2015; Thomson-Luque *et al.*, 2017). The most commonly used methods include differential centrifugation, filtration, cellulose column filtration, magnetic separation, and flow cytometric isolation. Among these, filtration is particularly suitable for *in vitro* antimalarial drug sensitivity experiments as it is simple to

perform, and leukocyte removal is efficient (Janse *et al.*, 1994; Lu *et al.*, 2011). As currently available filters are relatively expensive, however, a cheaper effective alternative is desirable. Filtration by cellulose powder column is cost-effective, but is time consuming not only in preparation, but also in the filtration process. Moreover, when dealing with small volumes of mouse blood, the recovery rate of RBCs is often low, as it is affected by column bed volume, dilution and wash conditions.

We previously showed that a prototype NWF filter was suitable for the treatment of *Plasmodium vivax*-infected human blood, with WBC removal rates similar to cellulose column filtration, but with the advantage that RBC recovery rates were significantly higher (Tao *et al.*, 2011). In addition, the NWF filter method was as fast and simple as Plasmodipur™ filtration; with the added benefit that filtered parasites remain viable (Li *et al.*, 2017; Lu *et al.*, 2017).

Here we have shown that the NWF filter can be used to deplete leucocytes from the blood of *P. berghei*-infected BALB/c, C57, Kunming mice and SD rats. Greater than 99.5% leukocytes were removed from these four species of rodent blood samples after treatment with a NWF filter. PCR semi-quantification of the BALB/c mouse housekeeping gene GPADH showed a 100-fold decrease in copy number estimation in filtered blood compared to unfiltered blood.

Both WBC counts and PCR amplification revealed the high efficiency of WBC removal from *P. berghei*-infected rodent animal blood using a NWF filter. The RBC recovery rate for rodent blood following NWF filtration was greater than 80.0%, lower than that previously achieved with *P. vivax*-infected human blood (95.0%). The reason for this difference is due to differences in the initial blood sample volume used, with five times more blood used in the human malaria sample compared to that from mice. In the case of human blood sample, the amount of blood cells absorbed by the fabric pad had reached saturation, whereas this had not occurred in the case of the lower-volume rodent blood sample. In order to achieve and increased yield of recovered blood cells, a larger volume of eluent may be used.

Microscopy of Giemsa's solution stained blood films showed that as well as efficiently removing WBCs, rodent malaria parasites and canine *Babesia* parasites in the recovered iRBCs were morphologically similar before and after NWF filtration. The subsequent infection of mice with *P. berghei* iRBCs that had passed through the filter demonstrates maintenance of the parasite viability following filtration. The use of the NWF to remove WBCs from intraerythrocytic stage parasite-infected blood, is therefore, an efficient and cost-effective alternative to current purification methods.

Conflict of interest
The authors declare that they have no competing interests.

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