Research Article
The Comparison of PCR Kits for the Detection of Erythrocytic Parasites on Filter Paper

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Dried blood spot (DBS) based PCR was considered an inexpensive and feasible method for detecting pathogens in the blood. The DBS carrier filter paper and PCR kits are crucial for accurate diagnosis. We evaluated 4 types of filter papers and 20 PCR kits for DBS samples. The PCR detecting Plasmodium results showed that the minimum detection limit of the 4 filter papers was $1 \times 10^2$ parasites/μL, and the positive rates of 20 PCR kits ranged from 0% to 100%. PCR results were satisfactory for detecting Plasmodium falciparum (P. falciparum) and Plasmodium vivax (P. vivax) in archived DBS samples and Babesia gibsoni (B. gibsoni) in fresh pet DBS samples. Our results provided a useful reference for the detection of blood pathogens with DBS samples and direct PCR, especially for screening the cost-efficacy combination of filter paper and PCR kit in resource-limited areas.

1. Introduction

Plasmodium [1–3] and Babesia [4–8] are two major intraerythrocytic parasites causing human and animal infection, namely malaria and babesiosis. Both are life-threatening. Fast and accurate diagnosis of these diseases is crucial for the treatment of patients and to contain the spread of these vector-borne diseases. Microscopy is the most common method for laboratory diagnosis of blood parasitic infection, but low parasitemia infection may lead to misdiagnosis. Sometimes, the identification of species of Plasmodium or Babesia may not be possible due to morphological similarity. More importantly, Babesia is easily confused with early-stage Plasmodium [3, 6, 9, 10] and can lead to unsuccessful treatment with antimalarial drugs. PCR-based molecular diagnostic approaches are important supplementary methods for accurate diagnosis [11–15], but these methods are usually not feasible in resource-limited areas due to equipment requirements and cost.

Since the 1960s, dried blood spot (DBS) samples have been used in the clinical diagnostic analysis for blood testing, mainly for the screening of neonatal metabolic disorders and genetic abnormalities [16–20]. Many DBS-based experimental methods have been successfully developed, such as analysis methods for nucleic acids, lipids, and other substances. Qualitative and quantitative detection based on DBS PCR has been widely used in the screening of infectious diseases such as HIV, HBV, CMV, and so on. It is also of great importance in the detection of parasitic infections in the blood [21–24], especially for Plasmodium infection [1, 9, 25, 26].
Collecting whole blood on filter paper as dried blood spot samples was optimal for the storage and transportation of blood samples from the field site to the laboratory for centralized testing [17]. As a sampling tool for PCR molecular diagnosis and surveillance, the quality of filter paper is particularly important. Among many types of filter paper, Whatman 903 filter paper has FDA approval and is widely used. Currently, 903 filter paper is provided as a relatively expensive ready-to-use sampling card. Whatman CF12 filter paper is a cheaper form of square sheet filter paper and is currently listed as one item of 903 Proteinsaver cards by its new manufacturer Cytiva. CF 12 filter paper is globally available and marketed as 903 filter paper in China. However, a comparison of physical properties and molecular detection performance of CF12 and other filter papers was not usually done.

At present, in order to facilitate the rapid control of many infectious diseases, there is a need for efficient and convenient nucleic acid collection and detection methods in the field. Existing PCR test requires a long detection time, and samples such as throat swabs and blood need to be extracted for nucleic acid before testing, which increases the overall diagnosis time. Moreover, the cost of nucleic acid extraction and detection is high, and some underdeveloped regions do not have proper nucleic acid extraction equipment and laboratory settings. Therefore, there was an urgent need for the development of rapid detection methods.

Direct PCR is one of the most valued rapid molecular detection techniques. Before 1993, direct PCR was used in the field of microbiology, for which the sample processing is very simple [19]. For example, colony PCR is a typical rapid method for screening colonies. Most direct PCR is based on the use of a genetically modified DNA polymerase, which makes it possible for bypassing nucleic acid extraction before PCR amplification, saving experimental materials and time. Moreover, it reduces the possibility of human error and sample cross-contamination [1]. The requirements for polymerase are high, not only in the tolerance to interferential components but also in the compatibility with the buffer of PCR reaction.

There are potent PCR inhibitors in crude samples such as blood, which can cause false negative PCR results [27]. Several major inhibitory components in blood have been characterized, that is, hemoglobin, immunoglobulin G, and lactoteferrin. The mechanism is related to the inactivation or inhibition of Taq DNA polymerase, which may reduce the efficiency and usability of direct PCR based on filter paper blood samples [28, 29].

We purchased four types of filter papers commercially available in mainland China and evaluated them in terms of weight, thickness, blood absorption performance, positive rates, and price. A total of 20 commercially available PCR kits, mainly for direct PCR of blood samples, were selected and tested using DBS samples. The aim of this research is to evaluate the cost-effectiveness of DNA polymerases that were suitable for molecular diagnosis of blood infection in DBS samples.

2. Materials and Methods

2.1. Filter Papers. Whatman CF12 (GE healthcare, UK), Advantec 545 (Advantec Group, Tokyo, Japan), and Jesiman Filter Paper (Jesiman New Material Co. Ltd., Wuhan, China) were purchased from the Taobao platform in China (https://taobao.com), and Gel Blot Paper was purchased from Sangon Biotech Co. Ltd. (Shanghai, China). Supplementary Table S1 details this information.

2.2. The Preparation of DBS Filter Paper Samples Containing Plasmodium parasites. Collect EDTA-K2 anticoagulated blood from mice infected with P. yoelii. Adjust the parasitemia to $1 \times 10^5$ parasites/$\mu$L, and 1, $10^1$, $10^2$, $10^3$, $10^4$, and $10^5$ parasites/$\mu$L blood samples were obtained by tenfold serial dilution with EDTA-K2 anticoagulated whole blood. Blood was absorbed by four types of filter papers to prepare DBS samples, each spot contains 50 $\mu$L of blood. The blood spot preparation schematic diagram is shown in Figure 1.

Plasmodium falciparum 3D7 (P. falciparum) dried blood spots: four levels of parasite density blood samples (1 and $10^1$ to $10^3$ parasites/$\mu$L) were obtained by tenfold serial dilution and absorbed on CF12 filter paper.

The DBS samples were placed on a clean bench and dried for 48 h. The filter papers were stored in a sealed plastic bag with silica gel desiccant.

2.3. Self-Made Blood Collection Card. The self-made blood collection cards are made with CF12 filter paper, consisting of upper and lower cover and enclosed sampling filter (see Supplementary Figure S1). It was placed upright to dry the blood samples after collection and to protect the DBS during transportation. Each DBS sampling card was individually packaged with silica gel desiccant in a plastic bag and stored at room temperature.

2.4. DBS Samples of Malaria-Infected Blood. Our laboratory has established a repository of malaria samples obtained over decades of research. Preserved DBS samples of Plasmodium vivax (P. vivax) infected blood were collected in Wuhe County, China, between 2009 and 2014, stored at -20°C. The DBS samples of P. falciparum-infected blood were collected from imported cases mostly from African countries, between 2010 and 2013, and stored at room temperature.

2.5. Animal Blood Samples. Self-made blood collection cards were sent to the pet hospitals located in three cities: Bengbu, Changsha, and Nanjing. DBS samples were collected from dogs. After complete drying, DBS samples were sent back to our lab for detection of Babesia infection.
2.6. Pretreatment of Blood Spots before PCR Reaction. Two pieces of dried blood spots (equivalent to 3–5 µL of whole blood) were punched with a 1.5 mm puncher and placed in one PCR reaction tube. To remove the hemoglobin in the sample and dust floating on the surface of the blood spots, 70 µL of sterilized ultrapure water was added to each tube, and the reaction tubes were incubated at 50°C for 5 min, 21°C for 15 s, 50°C for 1.5 min, and 21°C for 15 s. The supernatant in the tubes was then aspirated and discarded.

2.7. Primers Used for PCR Detection of Pathogens. The target of nested PCR for *Plasmodium* was 18S rRNA. Genus-specific primers rPLU6 and rPLU5 were used for the first round of amplification, and the amplified products were used for the second round of amplification, in which the primers used were species-specific for *P. falciparum* and *P. vivax*. The target gene for the detection of *Babesia gibsoni* was 18S rRNA. The sequences of all primers used in this research are shown in Supplementary Table S2.

2.8. The Information of 20 Commercial DNA Polymerases. All 20 DNA polymerases were purchased from Labgic Technology Co. Ltd. (Hefei, China), and the purchasing information is shown in Supplementary Table S3.

2.9. PCR Conditions. For each PCR, all reagent components were added according to their product instructions. For different PCR, the detailed programs were listed in Supplementary Tables S4–S6. The annealing temperatures were set according to the Tm value of each primer pair, and the extension times are set according to the expected fragment length and the enzyme elongation speed. All PCR reactions were carried out on an S1000 Thermal Cycler (Bio-Rad, USA).

3. Results

3.1. The Characteristics of Four Types of Filter Papers. We compared the general properties of the four types of filter papers available from the mainland China market, including thickness, weight, and blood absorption ability. The results showed that there were differences in the absorption time and the diameter of the blood spots when using healthy human blood and laboratory mice blood. For the absorption time of 50 µL whole blood by different filter papers, CF12 and 545 filter papers only take 3–5 seconds, which was significantly faster than the other two filter papers. CF12 and 545 filter papers formed more regular blood spots and were less prone to leakage (see Table 1). The Sangon Gel Blot paper could not completely absorb 50 µL of blood in a short time, and the blood sample was seen to spread unevenly in the filter paper, while no similar diffusion and leakage occurred in the other three filter papers (see Figure 2).

3.2. Detection Limits and Positive Rates of Direct PCR on Four Types of Filter Papers. A high-fidelity polymerase (Tks Gflex, TAKARA R060Q) based PCR was used to determine the detection limit for *Plasmodium* in the dried blood spots of four filter papers. The detection target was a 134 bp fragment of *P. yoelii* 17XNL 18srRNA gene. The results showed that the minimum detection limit of all four filter papers was $1 \times 10^2 \text{parasites}/\mu\text{L}$.

For comparison of the efficiency of different filter papers for DBS-based PCR, we purchased 20 commercial DNA polymerases for PCR detection of erythrocytic parasites in the DBS sample. The *Plasmodium* genus-specific primer rPLU6/5 was used to amplify a 1,003 bp fragment of *P. yoelii* in the DBS sample. The results showed positive rates of four filter papers that ranged from 56.7% to 63.3% (see Table 2), and the chi-square test showed no significant difference between the four filter papers ($p > 0.05$).

3.3. The Results of 20 DNA Polymerases for Detection of *Plasmodium Genes* in DBS. For comparing the performance of 20 polymerases, serial diluted *P. yoelii* 17XNL DBS samples on four filter papers were tested. The results showed that the positive rates ranged from 0% to 100% (see Table 2). Five PCR kits can detect all *Plasmodium* samples with different parasite densities on four types of filter papers.

3.4. Cost-Effectiveness Analysis of 20 DNA Polymerases. In order to screen cost-effective DNA polymerases, both unit price and positive rate need to be considered. As well known, the less template DNA in samples, the more difficult to amplify, the cost-effective criteria were established according to the difficulty of amplification: 1 point for positive of $10^3$ parasites, 10 points for $10^2$ parasites, and 100 points for $10^1$ parasites (see Table 3). Eight DNA polymerases with positive rates from 80% to 100% were selected for the subsequent experiments.

3.5. Performance Comparison of DNA Polymerases for Detection of *P. falciparum*. Blood samples of cultured *P. falciparum* were tenfold serial diluted (1–$10^3$ parasites/µL) by normal mice blood and DBS samples were prepared using CF12 filter paper. Eight selected DNA polymerases were tested for detection of *P. falciparum* using nested PCR techniques. The first-round primers rPLU5/6 were genus-

![Figure 1: The scheme of the preparation of dried blood spot samples by tenfold serial diluting *Plasmodium yoelii*-infected blood.](image)
specific, and the second-round primers rFAL1/2 were *P. falciparum*-specific. One microliter of first-round PCR product was used as the template DNA in the second-round PCR. The results showed that the following five DNA polymerases: Beyotime HemoTaq, NEB Hemo Klen Taq, TAKARA MightyAmp, TOYOBO KOD FX Neo, and Sangon Direct PCR Kit, were better for *P. falciparum*, with all positive rates at 100% (detailed amplification results are listed in Supplementary Table S7). These five DNA polymerases were selected for subsequent detection of clinical samples.

### 3.6. Direct PCR Results of Human Malaria Parasites in DBS Sample

Forty-one positive DBS samples of human malaria were retrieved from our laboratory archive and were analyzed by direct PCR using five cost-effective polymerases. The original filter papers used for preserving blood samples were not uniform: some of them were obvious thinner or thicker; some were prepared with an FTA card. Also, the density of parasites in each DBS sample was unclear. These samples consisted of 21 *P. falciparum* and 20 *P. vivax*. The results showed that the following three polymerases can detect both malaria parasites at 100% positivity rates: TAKARA MightyAmp, TOYOBO KOD FX Neo, and Sangon Direct PCR Kit. The regions from which these samples were taken and the results of the amplification of the five DNA polymerases are shown in Supplementary Table S8.

### 3.7. DBS-Sample-Based Direct PCR for Detection of Babesia in Dog Blood

In order to verify whether DBS direct PCR is suitable for detecting *Babesia* in dog blood, self-made CF12 blood collection cards were used to collect dog blood in three regional veterinary hospitals. DBS samples were sent back to our laboratory for the batch test. After receiving the samples, the direct PCR test was conducted by using only the MightyAMP kit. The results showed that two positive samples were detected from Bengbu dog blood DBS samples. Sequencing of PCR product and Giemsa’s stain confirmed that the two dogs were infected with *Babesia gibsoni*. The detailed results are shown in Supplementary Table S9.

### 4. Discussion

Due to limited resources in many tropical countries, it may not be possible to meet the basic storage requirements for blood samples for laboratory diagnostics. Many studies have shown that a variety of substances were stable in DBS and could be preserved for longer periods than traditional methods. DBS was considered an alternative to blood samples for the detection of antibodies or nucleic acid in resource-limited areas and in populations where venous blood collection, preservation, and transportation were difficult. However, differences in the nature of the filter papers could influence the analytic results, and the compatibility with key reagents and their uneven performance can also cause variable results of a DBS-based assay.
| DNA polymerases                | Whatman CF12 (parasites/µL) | Advantec 545 (parasites/µL) | Gel Blot paper (parasites/µL) | Jesiman Filter Faper (parasites/µL) | Positive rates of 20 polymerases |
|-------------------------------|-----------------------------|----------------------------|-------------------------------|-------------------------------------|----------------------------------|
| Beyotime | HemoTaq                      | +                          | +                             | +                                  | 83.30% (10/12)                   |
|             | Taq                          | –                          | –                             | +                                  | 50.00% (6/12)                    |
|             | HemoTaq HF                   | –                          | –                             | –                                  | 8.30% (1/12)                     |
|             | BeyoFusion                   | –                          | –                             | –                                  | 0.00% (0/12)                     |
| Sangon biotech | Direct PCR kit               | +                          | +                             | +                                  | 100.00% (12/12)                  |
|             | Taq Plus                     | –                          | –                             | –                                  | 0.00% (0/12)                     |
| TAKARA     | Tks Gflex                    | +                          | +                             | +                                  | 91.70% (11/12)                   |
|             | Taq                          | +                          | +                             | +                                  | 83.30% (10/12)                   |
|             | MightyAmp                    | +                          | +                             | +                                  | 100.00% (12/12)                  |
| TOYOBO      | rTaq                         | –                          | –                             | +                                  | 41.70% (5/12)                    |
|             | KOD Plus Neo                 | –                          | +                             | +                                  | 75.00% (9/12)                    |
|             | KOD FX Neo                   | +                          | +                             | +                                  | 100.00% (12/12)                  |
| TIANGEN     | Mouse Tissue Direct PCR kit  | –                          | +                             | +                                  | 83.30% (10/12)                   |
|             | Ultra                        | +                          | +                             | +                                  | 100.00% (12/12)                  |
| FOREVERSTAR | Blood direct PCR kit         | –                          | –                             | –                                  | 0.00% (0/12)                     |
| Thermo Fisher | Phire Hot Start II           | –                          | +                             | +                                  | 58.30% (7/12)                    |
|             | Platinum direct PCR kit      | –                          | +                             | +                                  | 58.30% (7/12)                    |
| NEB         | Hemo klen                    | +                          | +                             | +                                  | 100.00% (12/12)                  |
|             | Taq                          | –                          | –                             | –                                  | 0.00% (0/12)                     |
|             | Vent                         | –                          | –                             | –                                  | 83.30% (10/12)                   |
| Positive rates of 4 filter papers | 45.00% (9/20)     | 70.00% (14/20)              | 75.00% (15/20)                  | 35.00% (7/20)                     | 56.70% (34/60)                   |
|             |                             | 70.00% (14/20)              | (15/20)                        | 35.00% (7/20)                     | (36/60)                         |
|             |                             | 60.00% (12/20)              | (15/20)                        | 60.00% (14/20)                    | (38/60)                         |
|             |                             | 75.00% (15/20)              | (15/20)                        | 60.00% (12/20)                    | (36/60)                         |
|             |                             | 60.00% (12/20)              | (15/20)                        | 63.30% (38/60)                    | (38/60)                         |
|             |                             | 75.00% (15/20)              | (15/20)                        | 65.00% (13/20)                    | (38/60)                         |
|             |                             | 45.00% (9/20)               | (15/20)                        | 80.00% (16/20)                    | (38/60)                         |
In this study, four commercially available filter papers were compared in terms of their performance in absorbing blood, price, and suitability for nucleic acid detection using 20 DNA polymerases. The Whatman CF12 and Advantec 545 filter papers exhibited excellent blood absorption properties and were more convenient for preparation but were relatively expensive. The comparison of nucleic acid detection performances showed that there was no significant difference in the positivity and detection limits between the two filter papers. And the results from the archived DBS samples showed that all five DNA polymerases had high detection rates, and the three DNA polymerases, TAKARA Tsq Flex, TOYOBO KOD FX Neo, and Sangon Direct PCR Kit, gave better results and were more resistant to inhibitors in the blood. However, some polymerases may not be suitable for DBS PCR. Furthermore, the experimental results of direct PCR on archived human malaria DBS samples showed that all five DNA polymerases had high detection rates, and the three DNA polymerases, TAKARA MightyAmp, TOYOBO KOD FX Neo, and Sangon Direct PCR Kit, had 100% positive rates for both human malaria tests.

Table 3: Cost-effectiveness comparison of 20 DNA polymerases.

| DNA polymerases | Item no. | Price (USD) | Tests | Price/test (USD) | Positive rate (n/12) (%) | Scores* | Scores (price/test) |
|----------------|----------|-------------|-------|------------------|--------------------------|---------|---------------------|
| NEB Hemo klen Taq | M0332S | 186.55 | 200 | 0.93 | 100.00 | 444 | 476.00 |
| TAKARA MightyAmp | R071Q | 40.95 | 40 | 1.02 | 100.00 | 444 | 433.69 |
| Ultra HiFidelity PCR kit | KP203 | 97.28 | 80 | 1.22 | 100.00 | 444 | 365.14 |
| TOYOBO KOD FX Neo | KFX-201S | 31.38 | 20 | 1.57 | 100.00 | 444 | 282.98 |
| Sangon Direct PCR kit | B639289-0050 | 82.37 | 50 | 1.65 | 100.00 | 444 | 269.51 |
| TAKARA Tsq Flex | R060Q | 75.47 | 80 | 0.94 | 100.00 | 344 | 364.65 |
| TAKARA Taq | R001 A | 18.83 | 200 | 0.09 | 83.30 | 244 | 2,591.88 |
| Beyotime Hemo Taq | D7241S | 39.70 | 200 | 0.20 | 83.30 | 244 | 1,229.35 |
| Mouse Tissue Direct PCR kit | KG205 | 62.76 | 50 | 1.26 | 83.30 | 244 | 194.39 |
| NER Q5 | M0491S | 137.92 | 100 | 1.38 | 83.30 | 244 | 176.92 |
| TOYOBO KOD Plus Neo | KOD-401S | 23.54 | 20 | 1.18 | 75.00 | 144 | 122.37 |
| Phire Hot Start II | F122S | 220.76 | 200 | 1.10 | 58.30 | 34 | 30.80 |
| Platinum Direct PCR kit | A44647100 | 136.97 | 100 | 1.37 | 58.30 | 34 | 24.82 |
| Beyotime Beyotime Taq | D7205 | 4.39 | 160 | 0.03 | 50.00 | 24 | 874.08 |
| TOYOBO rTaq | TAP-211 | 23.54 | 100 | 0.24 | 41.70 | 14 | 59.49 |
| Beyotime Hemo Taq HF | D7243S | 47.85 | 200 | 0.24 | 8.30 | 1 | 4.18 |
| Beyotime BeyoFusion | D7220 | 16.95 | 80 | 0.21 | 0.00 | 0 | 0.00 |
| Sangon Taq Plus | B50013-0100 | 3.45 | 200 | 0.17 | 0.00 | 0 | 0.00 |
| StarLighter Blood Direct PCR | FS-P6001 | 18.83 | 500 | 0.04 | 0.00 | 0 | 0.00 |
| NER Vent | M0254 V | 61.03 | 200 | 0.31 | 0.00 | 0 | 0.00 |

*Scores were calculated based on the PCR results of 20 polymerases: 1 point for positive of 10^3 parasites, 10 points for 10^2 parasites, and 100 points for 10^1 parasites.

In order to perform large-scale screening, the cost per test must be taken into account. By scoring the PCR results to compare the cost-efficiency of direct PCR reagents, the method used in this study can be used as a useful reference for regions with limited resources to evaluate kits that were available locally and to facilitate large-scale screening of pathogens. In the reaction system for PCR, we followed the product instructions without modification. In fact, after optimization of direct PCR conditions for each primer and template, the performance of polymerases may be improved to some extent. This study suggests that a simple PCR nucleic acid detection technique based on filter paper and direct PCR kits with high sensitivity is suitable for the detection of pathogen genes within the blood, especially in resource-limited epidemic areas, and provides an economical and convenient option for large-scale screening of human and animal...
infectious disease by collecting filter paper blood samples in field sites and transporting them back to laboratories that are equipped to perform bulk testing.

Data Availability
The data that support the findings of this study are available in the manuscript as well as in the supplementary materials of this article.

Ethical Approval
All animal experiments were approved by the Experimental Animal Management and Ethics Committee of Bengbu Medical College, Bengbu, China (BBMC2021-195). The usage of archived dried blood samples was approved by the Institutional Review Board of Wuhan Center for Disease Control and Prevention (WHCDCIRB-K-2021013), Chenzhou Center for Disease Control and Prevention (20211218), Jiangsu Institute of Parasitic Diseases (IRB00004221), and the Biomedical Ethics Committee of Anhui Medical University.

Conflicts of Interest
The authors declare that there are no conflicts of interest.

Authors’ Contributions
Zhi-yong Tao and Qiang Fang contributed to conceptualization, methodology, writing, editing, validation, and resource; Pei-yi Zhang contributed to data acquisition, methodology, writing, data curation, and visualization; Lu Zhang, Chun-cao Li, and Rui Hu contributed to sample processing and data acquisition; Han-wu Zhu, Bei Zhou, Kai Wu, Ling-xu Li, and Da-wei Yao contributed to sample collection and resource; Yu-jie Cao, Dao-jin Wang, Chen-chen Zheng, and Run-qi Fang contributed to sample processing and sample collection; Xiu-min Hua, Yi-xuan Ni, Xiao-xia Jin, and Hui Xia contributed to reviewing and validation. Zhi-yong Tao and Pei-yi Zhang contributed equally.

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Supplementary Materials
The supplementary materials contain nine tables showing filter papers and DNA polymerases purchasing details; sample collecting information; and PCR primers, conditions, and results. In addition, Supplementary Figure S1 shows the self-made blood collection card based on CF12 filter paper. (Supplementary Materials)

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