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# COMPARISON OF SIX COMMERCIALLY-AVAILABLE DNA POLYMERASES FOR DIRECT PCR

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## SUMMARY

The use of a "direct PCR" DNA polymerase enables PCR amplification without any prior DNA purification from blood samples due to the enzyme's resistance to inhibitors present in blood components. Such DNA polymerases are now commercially available. We compared the PCR performance of six direct PCR-type DNA polymerases (KOD FX, Mighty Amp, Hemo KlenTaq, Phusion Blood II, KAPA Blood, and BIOTAQ) in dried blood eluted from a filter paper with TE buffer. GoTaq Flexi was used as a standard DNA polymerase. PCR performance was evaluated by a nested PCR technique for detecting *Plasmodium falciparum* genomic DNA in the presence of the blood components. Although all six DNA polymerases showed resistance to blood components compared to the standard Taq polymerase, the KOD FX and BIOTAQ DNA polymerases were resistant to inhibitory blood components at concentrations of 40%, and their PCR performance was superior to that of other DNA polymerases. When the reaction mixture contained a mild detergent, only KOD FX DNA polymerase retained the original amount of amplified product**.** These results indicate that KOD FX DNA polymerase is the most resistant to inhibitory blood components and/or detergents. Thus, KOD FX DNA polymerase could be useful in serological studies to simultaneously detect antibodies and DNA in eluents for antibodies. KOD FX DNA polymerase is thus not limited to use in detecting malaria parasites, but could also be employed to detect other blood-borne pathogens.

**KEYWORDS:** Blood direct PCR; Blood pathogen; Filter paper; DNA polymerase; PCR diagnosis; Field survey.

## INTRODUCTION

Mutational alteration of DNA polymerases to render them resistant to inhibition by blood components led to the development of "direct PCR" methods for the analysis of blood and soil samples[5]. Recently, various DNA polymerase kits have become commercially available for use in amplifying DNA directly from whole blood. During introduction of direct PCR experiments in our laboratory, we noticed a striking difference in blood-resistant performance between several kits. However, no studies have been conducted to evaluate these differences. We therefore compared the PCR performance of six commercially-available direct PCR-type DNA polymerases against a standard Taq DNA polymerase in the presence of PCR inhibitors found in blood components using a diagnostic nested PCR method for the detection of *Plasmodium* species genomic DNA.

Due to the limited infrastructure in many tropical countries, storage of blood samples for laboratory diagnosis is logistically complicated. Filter papers are often used as a practical means of sampling, storing, and transporting blood samples for the detection of blood pathogens such as *Plasmodium falciparum*[2,4]. The utility of filter paper blood samples for the measurement of serum antibodies and diagnostic PCR analyses has also been demonstrated[3]. Thus, we used blood samples eluted from dried blood on filter papers to which was added exogenous purified *P. falciparum* genomic DNA to examine the PCR performance and inhibitor resistance of the commercial DNA polymerases.

## METHODS

**DNA polymerases for direct PCR.** The six commercially-available direct PCR-type DNA polymerases examined in this study were purchased from the following suppliers: KOD FX, Toyobo (Tokyo, Japan); MightyAmp, Takara bio (Tokyo, Japan); Hemo KlenTaq, New England Biolabs (Ipswich, MA, USA); Phusion Blood II, Thermo Fisher Scientific (Hudson, NH, USA); KAPA Blood, KAPA Biosystems (Woburn, MA, USA); BIOTAQ, Bioline (London, UK). Non-direct PCR-type standard Taq DNA polymerase (Go Taq Flexi, Promega (Madison, WI, USA)) was used as a control.

**Preparation of PCR inhibitory blood components.** Filter papers (ADVANTECH, Tokyo, Japan) containing dried blood obtained from two healthy Japanese volunteers were cut into several 2.5-mm diameter disks. The blood was eluted by placing each disk in a tube containing 20 μL of TE buffer (10 mM Tris-HCl (pH8.0), 0.1 mM EDTA)[1] or a PBS-based elution buffer containing 0.05% Tween 20 and 0.05% sodium azide as used in simultaneous serological and PCR analyses[3]. The tubes were then

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heated for 15 min at 50°C, after which the disks were pressed gently to the bottom of the tube several times using a new pipette tip for each disk, and then heated for 15 min at 97 °C. The tubes were centrifuged at 15,000 rpm for 5 min and 1~8 µL of the supernatant (5%~40% blood eluent) was used in the 20-µL PCR reaction.

**PCR cycling conditions and primers.** A slightly modified standard nested PCR protocol was used to detect genus-specific *Plasmodium* genomic DNA within the highly conserved regions of the small-subunit rRNA gene[6,7]. The following primers, modified to increase sensitivity, were used: rPLU1-MOD1/rPLU5-MOD2 for nest 1 and rPLU3-MOD3/ rPLU4-MOD4 for genus-specific nest 2 amplifications; rPLU1-MOD1: GCTTGTCTCAMAGATTAAGCCATGCAAGTGA; rPLU5-MOD2: CACAGACCTGTTGTTGCCTTAAACTTCC; rPLU3-MOD3: TTTTTWHTATAAGGATAACTACGGAAAAKCTGTAGCTAATAC TTG; rPLU4-MOD4: TACCCGTCATAGCCATGTTAGGYCAATACC. Changes in the above nucleotide sequences are underlined. Details regarding the PCR mixture used in this study are summarized in Table 1.

To ensure maximal performance of each DNA polymerase, the PCR conditions recommended by each respective supplier were employed (Table 2). In case of PCRs using the Phusion DNA polymerase, we employed the PCR protocol for whole blood. Except in the case of negative controls, purified *P. falciparum* (strain 3D7) genomic DNA (2 ng) was added to the reaction mixture to serve as the template. For all DNA polymerases tested, the nest 2 reaction was performed in a similar manner using the nest 1 product (2 µL), with the exception of the annealing temperature, which was 58 °C.

All PCR assays were performed using a DNA Thermal Cycler 9700 (Applied Biosystems, Foster City, CA) with a standard ramp mode. Nest 2 PCR products (5 µL) were analyzed by gel electrophoresis on 3% agarose gels stained with ethidium bromide. Densitometric analysis (NIH ImageJ software) was used to determine the relative level of amplified target DNA. Amplified target DNA produced at more than 80% of the relative densitometric value of the positive control (PCR without blood components) were considered indicative of blood component-resistance.

## RESULTS

While the non-blood direct DNA polymerase (Go Taq Flexi, Promega) did not amplify the target gene region in the presence of blood components, all blood-direct DNA polymerases were resistant to blood components and produced the target PCR product in reaction mixtures containing as much as 10% blood eluent (Fig. 1). No prominent differences in the PCR results were observed between blood donors.

Both the KOD FX and BIOTAQ DNA polymerases were resistant to the inhibitory effects of blood components in 40% blood eluent reaction mixtures, whereas the intensity levels of the target band as compared to the positive control in each blood eluent were 83.8% and 111.1% for KOD FX and 43.0% and 85.5% for BIOTAQ, respectively.

**Table 1**
Final composition of PCR mixtures used in this study

The concentrations of nest 1 and 2 were identical. Each 20-µL reaction mixture for nest 1 amplifications contained 2 ng of *P. falciparum* genomic DNA in the absence or presence of 5%, 10%, 15%, 20%, or 40% blood eluent (or 40% elution buffer). Two microliters of the nest 1 amplification product were used as the DNA template for each of the 20-µL amplifications.

| | KOD FX | MightyAmp | Hemo KlenTaq | Phusion Blood | KAPA Blood | BIOTAQ | Go Taq |
|---|---|---|---|---|---|---|---|
| 5'-primer | 0.3 µM | 0.3 µM | 0.3 µM | 0.5 µM | 0.5 µM | 0.5 µM | 0.5 µM |
| 3'-primer | 0.3 µM | 0.3 µM | 0.3 µM | 0.5 µM | 0.5 µM | 0.5 µM | 0.5 µM |
| PCR buffer | 1x | 1x | 1x | 1x | ‡ | 1x§ | 1x |
| dNTP | 0.4 mM | * | 0.2 mM | * | ‡ | * | 0.2 mM |
| DNA polymerase | 0.02 units | 0.025 units | (1.6 µL)† | 1 unit | (10 µL)† | 0.5 units | 2 units |

*denotes dNTPs were included in the PCR buffer. †denotes indicated volume from each supplier. ‡denotes dNTPs and PCR buffer components were included in the DNA polymerase mixture vial. §denotes that the PCR buffer was supplied by SHIMADZU Corporation.

**Table 2**
Nest 1 PCR conditions

| | KOD FX | MightyAmp | Hemo KlenTaq | Phusion Blood | KAPA Blood | BIOTAQ | Go Taq |
|---|---|---|---|---|---|---|---|
| Initial denaturation | 94 °C, 2 min | 98 °C, 2 min | 95 °C, 3 min | 98 °C, 5 min | 95 °C, 5 min | 95 °C, 10 min | 95 °C, 2 min |
| Denaturation | 98 °C, 10 sec | 98 °C, 10 sec | 95 °C, 20 sec | 98 °C, 1 sec | 95 °C, 30 sec | 94 °C, 30 sec | 95 °C, 45 sec |
| Annealing | 55 °C, 30 sec | 55 °C, 15 sec | 55 °C, 30 sec | 55 °C, 5 sec | 55 °C, 30 sec | 55 °C, 1 min | 55 °C, 45 sec |
| Extension | 68 °C, 45 sec | 68 °C, 45 sec | 68 °C, 45 sec | 72 °C, 45 sec | 72 °C, 45 sec | 72 °C, 45 sec | 72 °C, 45 sec |
| Final extension | 68 °C, 7 min | 68 °C, 7 min | 68 °C, 10 min | 72 °C, 1 min | 72 °C, 10 min | 72 °C, 7 min | 72 °C, 5 min |

Thirty-five cycles of PCR were performed using each DNA polymerase.

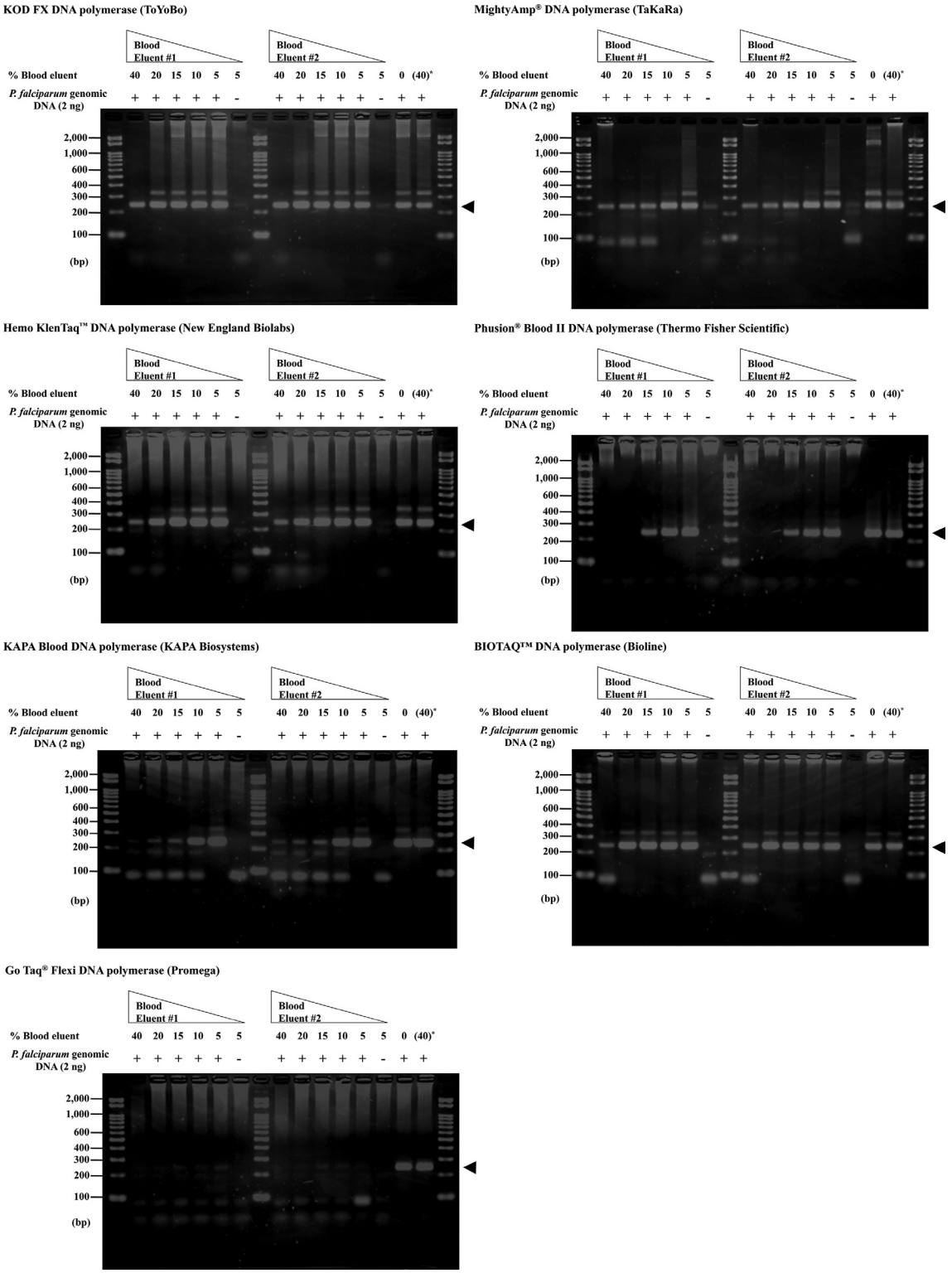


**Fig. 1 -** Analysis of the PCR performance of six commercially-available direct PCR-type DNA polymerases in the presence of 5-40% blood components in the reaction mixture. Nested PCR cycling conditions recommended by each enzyme's manufacturer were used. *Plasmodium falciparum* genomic DNA (2 ng) was detected by PCR using *Plasmodium* species detection primers in the presence of various concentrations of dried blood obtained from two apparently healthy Japanese volunteers that was eluted from filter papers (Left portion of each gel: eluents of blood from volunteer #1; right portion of each gel: eluents of blood from volunteer #2). Arrowheads indicate the target PCR product of the 18S ribosomal RNA gene region (240 bp). *Denotes elution buffer control (without blood components). The figure shows representative results from two independent experiments.

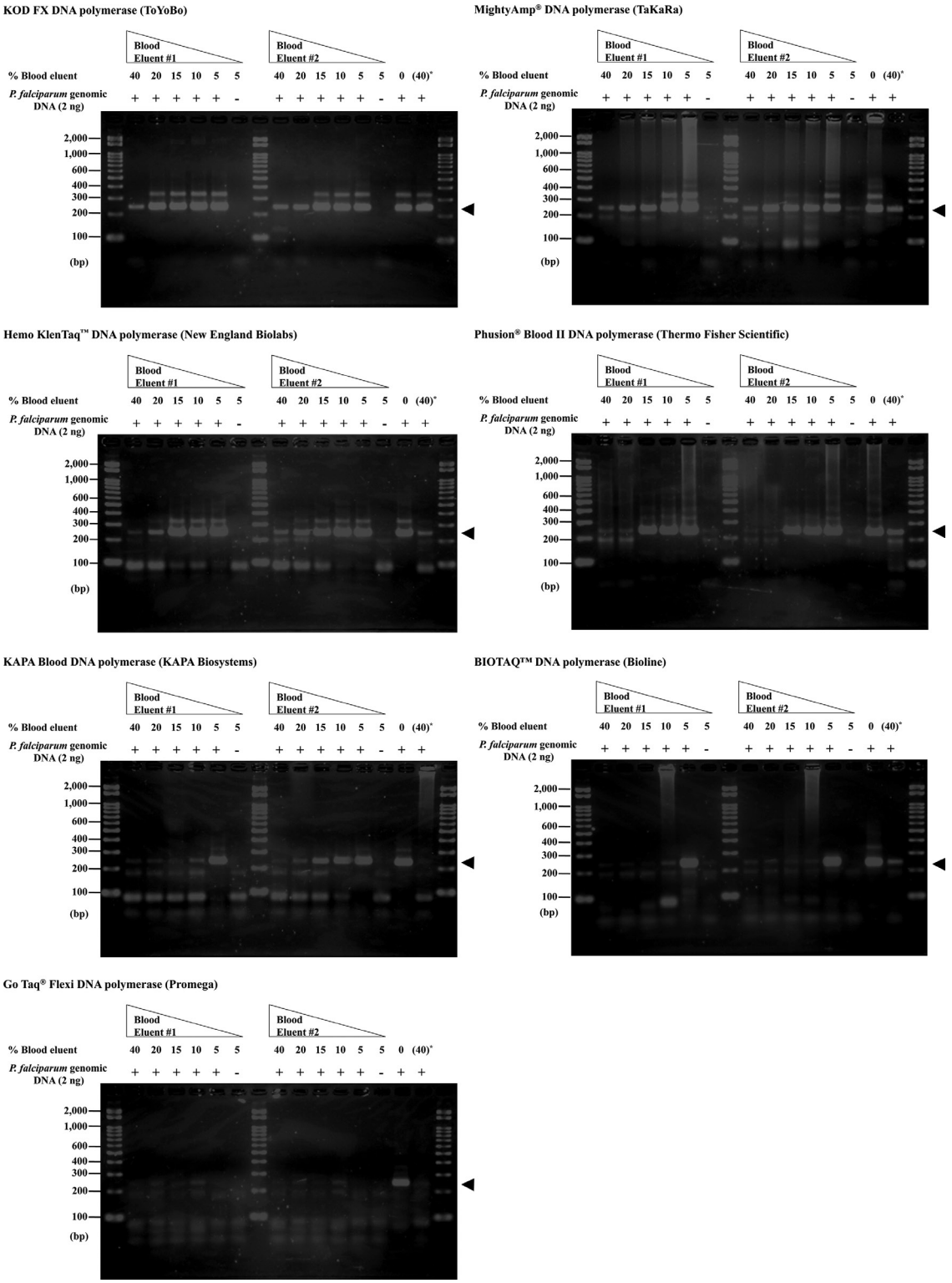


**Fig. 2 -** Analysis of the PCR performance of six commercially-available direct PCR-type DNA polymerases in the presence of 5-40% blood components and mild detergent (0.05% Tween20). PCR cycling conditions recommended by each enzyme's manufacturer were used. *Plasmodium falciparum* genomic DNA (2 ng) was detected by nested PCR using *Plasmodium* species detection primers in the presence of various concentrations of dried blood obtained from two apparently healthy Japanese volunteers that was eluted from filter papers (Left portion of each gel: eluents of blood from volunteer #1; right portion of each gel: eluents of blood from volunteer #2). Arrowheads indicate the target PCR product of the 18S ribosomal RNA gene region (240 bp). *Denotes elution buffer control (without blood components). The figure shows representative results from two independent experiments.

Hemo KlenTaq DNA polymerase was resistant to blood components at concentrations up to 20%, while Mighty Amp, Phusion Blood II, and KAPA Blood DNA polymerases were resistant to blood components at concentrations up to 10% of the reaction mixture (Fig. 1). These results suggest that of the six commercially-available DNA polymerases we tested, KOD FX DNA polymerase is the most resistant to blood component inhibitors.

Although some additional high-molecular-weight bands (over 2,000 base pairs) were produced using KOD FX DNA polymerase, no primer dimers (under 100 base pairs) were detected. On the other hand, BIOTAQ DNA polymerase produced no detectable high-molecular-weight additional bands, but the formation of primer dimers was observed in the negative control (without template DNA) and 40% blood eluent samples.

It has been shown that dried blood spotted onto filter paper is suitable for laboratory diagnostic procedures involving PCR analysis combined with immunoglobulin detection[3]. Therefore, we also examined the performance of the six commercially-available DNA polymerases in combined diagnostic tests using a phosphate buffered saline-based elution buffer containing a mild detergent (0.05% Tween 20). As shown in Figure 2, KOD FX DNA polymerase was resistant to blood components from one of the donors at concentrations up to 20%, and from the other donor at concentrations up to 15%. A DNA band of similar intensity level (88.5% as compared to the control band) was produced in the 40% elution buffer sample without blood components, suggesting that the activity of KOD FX DNA polymerase is not affected by Tween 20. The other DNA polymerases were not resistant to the 40% elution buffer containing 0.05% Tween 20, clearly demonstrating the superiority of the KOD FX DNA polymerase for assays of this type.

## DISCUSSION

It is both time-consuming and costly to determine which direct PCR DNA polymerase would be the most suitable for detecting a target gene from a specific pathogen. However, our data highlight the importance of comparing the performance of several DNA polymerases prior to establishing PCR conditions. The PCR fidelity of KOD FX DNA polymerase is reportedly more than 10-fold higher than that of standard Taq DNA polymerases, and similar to that of *Pfu* DNA polymerase. The high fidelity of KOD FX DNA polymerase results in more accurate PCR analyses[8,9].

Our results indicate that among the commercially available polymerases we tested, KOD FX DNA polymerase has the highest potential for resistance to blood components, making it the most suitable enzyme for attempting to detect the genomic DNA of pathogens in samples containing high concentrations of blood. Theoretically, employing DNA polymerases with higher resistance to blood components would enable researchers to perform PCR assays with sensitivity high enough for large-scale epidemiological studies. In addition, because of its resistance to mild detergents such as Tween 20, KOD FX DNA polymerase could be useful in serological studies to simultaneously detect both antibodies and DNA in eluents used in testing for antibodies.

However, KOD FX DNA polymerase retains 3' to 5' exonuclease activity, which potentially leads to a non-specific binding between primers and contaminated human genomic DNA (Toyobo web site: http://www.toyobo.co.jp/seihin/xr/lifescience/products/product/jisshirei/archives/2008/04/pcr3pcr.html). Therefore, we can not exclude the possibility of 3' to 5' exonuclease activity of the enzymes (KOD FX) leading to the non-specific band (240 bp) in the negative control (Fig. 1) was involved. In this line, KOD FX may not be suitable for PCR using primers such as targeting the ribosomal RNA gene.

In PCR protocols, we did not employ modifications of the recommendation of the manufacturers, which might give better results. Indeed, FUEHRER *et al*. reported a modified protocol for Phusion Direct PCR[4]. Such a modification method should be applied for each PCR enzyme to achieve more improved results.

The results of the present study suggest that the increased PCR sensitivity of KOD FX DNA polymerase may aid diagnostic field research efforts that involve the analysis of large numbers of samples for the presence of *P. falciparum* and other *Plasmodium* species. Our results suggest that this enzyme could also be employed for the detection of other blood-borne pathogens.

## RESUMO

### Comparação de seis polimerases de DNA disponíveis comercialmente para o PCR direto

O propósito deste estudo foi avaliar 6 polimerases de DNA disponíveis comercialmente que são resistentes aos inibidores do PCR para uma amplificação potencial de DNA de amostras de sangue total. O DNA genômico do parasita humano da malária, *Plasmodium falciparum,* foi analisado sob condições que incluíram os componentes inibidores do sangue extraído de sangue ressacado em papel de filtro. Nossos resultados sugerem que a polimerase KOD FX DNA é superior a outras polimerases.

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