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Effect of EDTA and ACD on blood storage for the detection of *Plasmodium falciparum* by PCR

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Blood samples are extensively used for PCR-based diagnosis of microbial infections and screening for blood banking[1]. It is not possible to do PCR with fresh blood samples each time. This preliminary study is aimed to compare the effect of blood storage (–20ºC) on PCR for detecting *Plasmodium falciparum* in the two most commonly used anticoagulants such as ethylenediaminetetra acetic acid (EDTA) and acid citrate dextrose (ACD)[2,3].

The study was conducted in Department of Microbiology, King George's Medical University (KGMU), Lucknow, Uttar Pradesh, India. The protocol was approved by the institutional ethical committee, KGMU, Lucknow. Informed consent was obtained from relatives of the patients.

Seven microscopy and rapid diagnostic test (RDT) positive blood samples for falciparum malaria, three microscopy and RDT negative (healthy persons) blood samples (Haemoglobin ≤12%) were collected in EDTA and ACD anticoagulants and stored at –20ºC for a period varying between 2 and 10 months.

These samples were collected before start of the antimalarial therapy during the period from August 2005 to April 2006. All patients had acute febrile illness and later they had responded to antimalarial therapy. Giemsa stained thick and thin blood films were observed under microscope. RDT for detecting the HRP-2 antigen of *P. falciparum* was performed using a commercially available kit (Paracheck Pf, Orchid Biomedical Systems, Goa, India) and results were interpreted as per manufacturer's instructions.

The protocols according to Patsoula *et al*[4], and Witoon *et al*[5], were followed on EDTA and ACD stored blood. Either of these methods in their exact format could not work in our settings. The reason may be that these authors had used fresh EDTA and sodium dodecyl sulphate blood. So we selected primer sets used by these authors but standardised PCR mix and PCR protocol in our laboratory which produced optimal results.

The rapid DNA extraction method according to Michael *et al*[6], and Raj *et al*[7] was selected with a few modifications. Briefly, 500 µl of ice-cold 5 mM sodium phosphate (pH-8) was added to 20 µl of venipuncture blood and vortexed. After centrifugation for 6 min/14500 rpm in a micro centrifuge tube at 4ºC, the supernatant was discarded. The pellet was suspended in 100 µl PBS containing 0.01% saponin and washed twice with same buffer by repeating the above steps of vortexing and centrifugation. After washing, the pellet was suspended in 50 µl of sterile

triple distilled water, vortexed and boiled for 20 min at 100°C. It was again vortexed before use for PCR. PCR was done separately for both sets of primers on all ten samples in the month of June 2006. Two primer sets were used as follows:

Primer set 1 (Patsoula *et al*[4])

PL3 (5' ATGGCC GTT TTT AGT TCG TG 3')
PL5 (5' ACG CGT GCA GCC TAG TTT AT 3')

Primer set 2 (Witoon *et al*[5])

PF1 (5' GCT ACA TATGCT AGT TGC CAG AC 3')
PF2 (5' CGT GTACCA TAC ATC CTA CCA AC 3')

For primer set 1, PCR reaction was performed in a mix, with a final volume of 50 µl containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 20 pm of each PL3 and PL5 primer 2.5 units Taq-DNA polymerase (Invitrogen, Brazil). For primer set 2 reaction mix was same as above except that primers used were PF1 and PF2.

In each run, an initial 4-min denaturation at 94°C was followed by 35 cycles, each of denaturation at 94°C for 30 sec, annealing at 52°C for 35 sec, extension at 72°C for 30 sec, and then a final extension at 72°C for 5 min. PTC100 (MJ Research, Watertown, mass model, USA) was used in all experiments. The PCR products were subjected to electrophoresis in 1.5% agarose gel (Fig. 1).

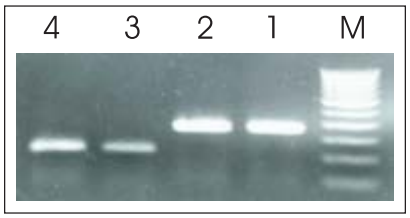

Fig. 1: Agarose gel electrophoresis pattern of PCR products from *P. falciparum* positive long-term stored blood samples. Lanes 1, 2 (primer set 1) and lanes 3, 4 (primer set 2) produced 346 bp and 206 bp with EDTA, ACD respectively, M–100 bp ladder

For primer set 1, EDTA stored blood gave 100% (7/7) sensitivity and 100% (3/3) specificity whereas ACD stored blood gave only 14.28% (1/7) sensitivity and 100% (3/3) specificity. For primer set 2, EDTA stored blood gave 57.14% (4/7) sensitivity and 100% (3/3) specificity, whereas ACD stored blood gave only 28.57% (2/7) sensitivity and 100% (3/3) specificity.

The results from our preliminary study showed that EDTA with primer set 1 produced positive results from blood samples stored as old as 10 months, whereas EDTA with primer set 2, produced positive results for blood samples old up to eight months only. These results along with the results of ACD (both sets of primers) are shown in Fig. 2.

This suggests that long-term EDTA stored blood (–20°C) when used with Patsoula *et al*[4], primer and our standardised PCR mixture and cycles produced results with 100% sensitivity and 100% specificity, however, there was decrease in sensitivity with ACD stored blood with both sets of primers. One of the

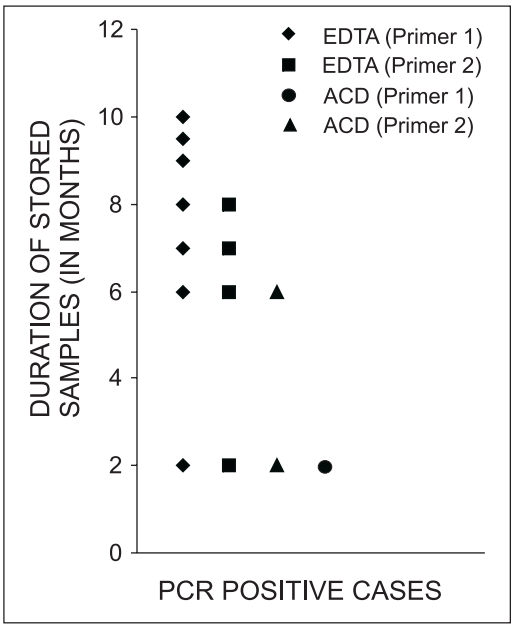

Fig. 2: Effect of duration of blood storage in EDTA and ACD anticoagulants on PCR for detecting *P. falciparum* malaria

reasons could be that the dilution effect caused by the liquid formulation of ACD decreased the nucleic acid recovery[8–10]. However, the exact reasons are not known.

So based on this preliminary study EDTA appeared to be a better anticoagulant than ACD for delayed blood processing in case of PCR analysis for *P. falciparum*. Our analysis, though, based on a small sample size, produced optimal results. However, a study is needed with a large number of stored blood samples to get a stronger evidence of the above results.

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