Short Report: Malaria Diagnosis by a Polymerase Chain Reaction–Based Assay Using a Pooling Strategy

Ajay R. Bharti,* Scott L. Letendre, Kailash P. Patra, Joseph M. Vinetz, and Davey M. Smith
University of California San Diego, La Jolla, California; Veterans Administration San Diego Healthcare System, San Diego, California

Abstract. Pooling clinical specimens reduces the number of assays needed when screening for infectious diseases. Polymerase chain reaction (PCR)-based assays can have 100-fold greater sensitivity, especially in the setting of low parasitemia or subclinical infections. Despite this advantage, the high cost of reagents, equipment, and quality assurance have limited PCR-based diagnostic assays primarily to research settings. Gal and others have shown that malaria DNA can be successfully detected in serum rather than the commonly used whole blood samples when using PCR-based assays. Previously, we showed that this applies to stored serum samples as well. This allows testing banked sera samples, a commonly stored specimen in clinical studies, to determine malaria infection rates in a sensitive manner. To further advance the usefulness of this technique, we sought to increase efficiency by pooling specimens.

Methods that pool clinical specimens before performing diagnostic tests have been shown to be an efficient way of screening for infectious diseases. This technique was first proposed by Dorfman to screen for syphilis in military recruits. Modifications to Dorfman’s original pooling algorithm by Finucan and Phatarfod and Sudbury have further improved the technique by reducing the number of individual tests needed to identify positive samples. Nucleic acid amplification tests using pooled specimens are now used for detecting viral infections such as acute HIV, hepatitis B and C, and West Nile virus, but not malaria.

For detecting malaria infection, we propose adapting the pooling platform described by WESTreich and others. One hundred individual clinical samples are arranged in rows and columns in a 10 × 10 matrix format (Figure 1). Ten individual samples are pooled by row and column such that each sample is represented in 2 of the 20 mini-pools, and each pool is assayed. Negative results of all 20 pools would exclude malaria infection in the 100 samples and preclude further testing. For a matrix with positive pools, additional assays may be needed to determine which sample(s) were positive, depending on the number and location of the individual positive samples. Sample selection for additional assays is guided by the 10 × 10 matrix platform. In our example (Figure 1), the four positive pools (black squares in rows D and F and columns 4 and 6) can result from various combinations of positive samples in the locations: D4, D6, F4, and F6 (gray and black circles). These four samples would require further testing; therefore, in this example, the pooling method would identify the two positive samples (black circles D4 and F6) using only 24 PCR assays (20 row and column pools + 4 individual) instead of the 100 needed by individual testing. In regions of even lower malaria prevalence, a master -pool comprised of all 100 samples by pooling the row or column pools (Figure 1), resulting in 1:100 dilution for a single positive sample, can also be used.

Our goals for this study were to (1) propose a strategy for pooling specimens and (2) show the feasibility of malaria detection by PCR-based methods using pooled, field-collected serum samples.

The details of specimen selection and cohort enrollment have been reported earlier. This study was approved by the Institutional Review Boards/Ethical Committees of the University of California San Diego, Universidad Peruana Cayetano Heredia, AB PRISMA, and US Department of Defense. Permission to conduct the study was provided by the Loreto Director of Health, Iquitos, Peru. Informed consent was obtained from all participants before enrollment. Blood (2–4 mL) was collected from participants with acute malaria infection and uninfected controls from Iquitos, Peru, who had Plasmodium vivax or P. falciparum parasitemia confirmed by microscopy. Serum was separated and stored at −20°C for an average period of 6 weeks. The samples were then stored at −80°C for an average of 4.5 years before use in this study. Sufficient serum was available from 15 participants with malaria diagnosed by light microscopy and 5 uninfected individuals.

The following semi-quantitative system was used: < 1 parasite/100 high power fields (HPF); +, 1 to < 2 parasites/HPF; ++, 2–20 parasites/HPF; +++, 21–200 parasites/HPF; ++++, > 200 parasites/HPF. Parasite concentration was also measured by determining the number of parasites per 200 white blood cells (WBCs). The parasitemia, as parasites per microliter, was calculated using a standard WBC count of 8,000/μL.

Two representative sets of 100 specimens each were prepared using malaria positive and negative sera. Because of limited serum samples, sufficient serum for testing in the two matrices was available for 6/15 microscopy-confirmed
malaria patients. Commercially available serum (Catalog no. 100–512, Gemini Bio-Products, West Sacramento, CA) was used as a substitute for malaria-negative sera. The first set was comprised of serum from one malaria-positive sample (1% prevalence) and the second set contained five malaria-positive samples (5% prevalence) with the remainder made up of 99 and 95 malaria-negative serum samples, respectively. Malaria-positive samples were randomly placed in the two matrices in a blinded fashion. Row and column pools were prepared using 100 μL serum from each sample, resulting in 20 pools of 1 mL each. From each pool, DNA was extracted from 400 μL of pooled sera using the DNeasy Blood and Tissue Kit (Catalog no. 69506, Qiagen, Valencia, CA) according to the manufacturer’s blood and body fluid protocol. DNA extracted from 200 μL of each undiluted malaria-positive serum sample was used as positive control. Similarly, DNA from 200 μL serum from cases that did not have malaria based on light microscopy and PCR was used as negative control.

The feasibility of detecting parasite DNA if there was only one positive sample in a pool was tested in pools of 10 (1:10 dilution) and 100 (1:100 dilution) serum samples. Row and column pools containing serum (100 μL) from single malaria positive case also included nine malaria-negative cases (900 μL pooled serum; Gemini Bio-Products). A master pool of 100 specimens was prepared by pooling 100 μL of serum from either the 10 row or column pools.

PCR amplification was performed using a modification of the technique originally described by Snounou and others6 with primers targeting the Plasmodium spp. 18S small subunit ribosomal RNA genes. The PCR reaction was performed in a total volume of 50 μL containing 20 μL extracted DNA, 25 μL of HotStar Taq Master Mix (Catalog 203443; Qiagen), and forward and reverse primers (0.2 μmol/L). Cycling conditions were incubation at 95°C for 15 minutes, followed by 35 cycles of 95°C for 30 seconds, and 58°C for 1 minute, with a final incubation at 72°C for 1 minute. Primer sequences were as follows: 5′-TTAAAATTTGTTGCAGTTAAACG-3′ (sense) and 5′-CCTTGTGTGCCTTAAAAACTTC-3′ (antisense). The presence of amplification products was detected by ethidium bromide staining after agarose gel (1.8%) electrophoresis.

Plasmodium vivax was the predominant infecting species in our study (12/15), with P. falciparum accounting for the remainder. The parasite counts, as measured by microscopy, were <1+ to +++ (mean, 2,704 parasites/μL; range, 1,000–7,407 parasites/μL) for subjects with P. vivax malaria and 1+ to ++ (mean, 7,778 parasites/μL; range, 2,210–16,730 parasites/μL) for subjects with P. falciparum malaria.

In the first set of 100 specimens, malaria PCR identified two positive pools (Figure 2A). The 10 × 10 matrix identified the individual sample (P. vivax parasitemia, 2,694 parasites/μL or +++) that resulted in the positive tests without any further testing. In the second set, there were nine positive pools (Figure 2B). The 10 × 10 matrix guided selection of individual samples for further PCR assays that identified the five malaria-positive samples (P. vivax parasitemia range, 234–7,407 parasites/μL or <1+ to +++)

The PCR-based assay detected parasite DNA in all 15 (100%) undiluted samples and in mini-pools with a 1:10 dilution (Table 1). This implies that all 15 samples would have tested positive in a 10 × 10 matrix. In master pools with a 1:100 dilution, 12/15 (80%) samples had detectable DNA, with a mean parasite count of 4,083 parasites/μL (range, 926–16,730 parasites/μL or + to +++). In the three negative samples, the mean parasitemia was 2,260 parasites/μL (range, 234–4,395 parasites/μL).
or < 1+ to ++). Results from the second matrix using DNA extracted from malaria-positive vertical and horizontal mini-pools and individual serum samples are shown in Figure 3. These PCRs were designed to maximize sensitivity for qualitative purposes (detection of presence or absence of malaria template), and quantitative conclusions cannot be made. All PCR assays were carried out in triplicate.

In this study, we evaluated, for the first time, a strategy that pools clinical samples to improve the efficiency of screening for malaria using a PCR-based assay. We also showed the feasibility of such methods for *P. vivax* and *P. falciparum* by using long-term cryostored serum samples (> 4 years), which could be important for the retrospective investigation of specimen banks of cohort studies.

The success of the proposed pooling strategy depends on the ability of PCR to detect parasite DNA despite the dilutional effect of pooling. The detection limit of malaria PCR is ≥ 5 parasites/μL, and in our experiments, the mini-pools with 1:10 dilution had parasite levels well above this (> 20 parasites/μL). Detectable parasite DNA was also present in all except 3 of 15 master pools with 1:100 dilution. Low parasitemia by microscopy may explain the false-negative PCR.

### Table 1

Results of microscopy and PCR detection of *Plasmodium* DNA using undiluted and pooled stored serum

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Species</th>
<th>Parasites/μL</th>
<th>Microscopy</th>
<th>PCR result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Undiluted (1:10 dilution)</td>
<td>Master-pool (1:100 dilution)</td>
</tr>
<tr>
<td>1</td>
<td>Pf</td>
<td>16,730</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Pv</td>
<td>1,942</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Pf</td>
<td>4,395</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Pv</td>
<td>1,000</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Pv</td>
<td>2,684</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Pv</td>
<td>2,150</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Pv</td>
<td>3,540</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Pv</td>
<td>7,407</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Pv</td>
<td>2,694</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Pv</td>
<td>4,210</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Pv</td>
<td>3,042</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Pv</td>
<td>2,614</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>Pv</td>
<td>926</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>Pv</td>
<td>234</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>15</td>
<td>Pf</td>
<td>2,210</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**PI = Plasmodium falciparum; PV = Plasmodium vivax.**

We recognize that there may be other clinical scenarios in which parasitemia may be so low as to give false-negative results during PCR-based screening with pooled samples, as in asymptomatic malaria infection and inadequate response to antimalarials caused by malarial drug resistance. However, the pooling strategy should still have increased sensitivity over microscopy, because experienced microscopists can detect as low as 20 parasites/μL, but routine diagnostic laboratories often have a much inferior sensitivity of detection (500 parasites/μL or 0.01% RBC infected).

Given the exploratory nature of our study, we would like to address its limitations. First, because of the small sample size, we cannot accurately assess the sensitivity and predictive values of the pooling strategy. These test characteristics will need to be determined in much larger studies and more appropriately in various clinical populations. Second, although this technique should be relatively easy to adapt for clinical care, it will need to be evaluated in the field with both serum and

---

**Figure 3.** PCR amplification products using *Plasmodium* genus-specific primers after DNA extraction from pooled and individual serum samples from matrix shown in Figure 2B. Lane 1, 100-bp DNA size marker ladder; Lanes 2–10, DNA extracted from malaria-positive vertical and horizontal mini-pools; Lanes 11–15, DNA extracted from malaria-positive individual specimens that constituted the mini-pools; Lane 16, DNA extracted from serum from a malaria-negative control; Lane 17, DNA extracted from *P. falciparum* (3D7) strain.
whole blood samples. In fact, the yield of parasite DNA from whole blood or dried blood spots is most likely to be greater than in serum, and these specimens may be better suited to pooling. Third, the cost-effectiveness of the proposed methods will need to be formally evaluated. In hypoendemic malaria regions (<10% prevalence), the mini-pooling strategy may provide significant cost-savings over screening samples using PCR-based assays individually or for screening asymptomatic individuals. In regions of even lower malaria prevalence, a master pool comprised of all 100 samples by pooling the row and column pools might be useful with considerable cost-savings. The optimal size of the pool would, therefore, depend on malaria prevalence in the region and the group of patients of interest, such as symptomatic versus asymptomatic.

In these studies, screening for malaria infection with PCR-based assays using pooling platforms is sensitive and efficient, although field studies using a variety of biological samples and in various malaria endemic populations are needed for validation and to evaluate its cost effectiveness.

Received May 22, 2009. Accepted for publication July 27, 2009.

Acknowledgments: The authors thank the patients from the city of Iquitos and the surrounding villages for their participation and our field staff for their assistance.

Financial support: A.R.B. is supported by National Institute of Mental Health Grant R25 MH81482 and the REACH (Research and Education in HIV/AIDS for Resource-Poor Countries) Initiative of Tibotec; J.M.V. is supported by a Doris Duke Charitable Foundation Innovations in Clinical Research Program grant, NIH/NIAID Grant K24AI068903, and NIH Fogarty International Center Global Infectious Diseases Training Grant 5D43TW007120; D.M.S. is supported by National Institutes of Health Grants MH083552, AI077304, AI09432, AI38858, AI43638, AI43752, AI29164, AI47745, MH625N12, AI047745, and AI57167 and the UCSF Center for AIDS Research (AI36214); and the HIV Neurobehavioral Research Center (HNRC) is supported by Center Award P30 MH62512 from the National Institute of Mental Health.

Authors’ addresses: Ajay R. Bharti, Scott L. Letendre, Kailash P. Patra, and Joseph M. Vinetz, University of California, San Diego, School of Medicine, Division of Infectious Diseases, Department of Medicine, 9500 Gilman Drive, 0847, La Jolla, CA 92093-0847. Davey M. Smith, Veterans Administration San Diego Healthcare System, San Diego, CA.

REFERENCES


