**BACKGROUND:** Malaria control programs have achieved remarkable success during the past decade. Nonetheless, sensitive and affordable methods for active screening of malaria parasites in low-transmission settings remain urgently needed.

**METHODS:** We developed a molecular screening method, capture and ligation probe-PCR (CLIP-PCR), which achieved the sensitivity of reverse-transcription PCR but eliminated the reliance on RNA purification and reverse transcription. In this method, 18S rRNA of genus *Plasmodium* is released from blood, captured onto 96-well plates, and quantified by the amount of ligated probes that bind continuously to it. We first used laboratory-prepared samples to test the method across a range of parasite densities and pool sizes, then applied the method to an active screening of 3358 dried blood spot samples collected from 3 low-endemic areas in China.

**RESULTS:** *Plasmodium falciparum* diluted in whole blood lysate could be detected at a concentration as low as 0.01 parasites/μL, and a pool size of ≤36 did not significantly affect assay performance. When coupled with a matrix pooling strategy, the assay drastically increased throughput to thousands of samples per run while reducing the assay cost to cents per sample. In the active screening, CLIP-PCR identified 14 infections, including 4 asymptomatic ones, with <500 tests, costing <$US0.60 for each sample. All positive results were confirmed by standard quantitative PCR.

**CONCLUSIONS:** CLIP-PCR, by use of dried blood spots with a pooling strategy, efficiently offers a highly sensitive and high-throughput approach to detect asymptomatic sub-microscopic infections with reduced cost and labor, making it an ideal tool for large-scale malaria surveillance in elimination settings.
mission episodes (1). In fact, a recent trial of large-scale community-wide screening by use of RDT in Burkina Faso found no impact of active case detection on parasite prevalence or incidence of clinical episodes after 12 months of follow-up (7), most likely because of the poor sensitivity of RDT to detect all parasitemic and gametocyteemic individuals (7). Thus, with current diagnostic technologies, active case detection is unlikely to be sufficient for malaria elimination. Sensitive molecular technology such as PCR testing provides a more accurate diagnosis than microscopy or RDT. However, widespread implementation of PCR has been limited by cost per test, the need for skilled labor, and (because of the requirement for nucleic acid extraction) the difficulty to scale up in throughput.

As parasite prevalence approaches elimination, a greater number of case detections have to be performed to find 1 positive infection. For example, in Yunnan province, China, approximately 350000 individuals were screened to find 460 infections in 2013. To encourage community-wide full participation, WHO proposed that all active screening services be free of charge in the elimination phase (8). For most programs, increased massive screenings have placed additional demands on human and financial resources, stretching a surveillance system already strained by high workload and limited funding. These challenges emphasize the critical importance of technology innovation in malaria elimination, requiring screening methods with higher throughput and lower cost, in addition to the high sensitivity required to detect asymptomatic subpatent infections. This need has been recognized as one of the top priorities of the field (1).

Here we describe the development of a novel technology, capture and ligation probe-PCR (CLIP-PCR), which effectively meets these challenges.

Material and Methods

STUDY AREAS

We selected 3 counties in 2 provinces, Gengma County and Tengchong County of Yunnan Province and Feidong County of Anhui Province, as survey fields according to ecological features as well as epidemic situations. The morbidity of malaria in Gengma, Tengchong, and Feidong counties in 2012 was 0.85, 3.00, and 0.07 per 100000 inhabitants, respectively, according to the local center for disease control and prevention.

FIELD SAMPLES FROM STUDY COHORTS

We collected 3358 blood smears and dried blood spots (DBSs) according to standard protocols from the 3 cohorts between May and October 2013. The first group consisted of 505 individuals from a village in Gengma County, including 226 local residents and 279 primary schoolchildren. Whereas 2 children had been diagnosed with Plasmodium vivax infection in the past 6 months, none of those tested in this study showed malaria-related symptoms on the day of sampling. Whole blood samples of this cohort were collected in EDTA or heparin tubes and stored at −20 °C until the quantitative PCR (qPCR) test. The second cohort included 2064 people in Tengchong County, including 914 migrant workers returning from malaria-endemic Myanmar. The third group consisted of 789 people in Feidong County, 69 of whom were migrant workers returning from malaria-endemic Africa.

Samples were collected with written informed consent. Ethics approval was granted by the institutional review boards of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, and National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention. Patients with positive microscopy or RDT were treated with antimalarial agents.

DETECTION OF 18S rRNA BY CLIP-PCR

Each 3-mm-diameter DBS punch (or 60 μL whole blood) was lysed with 100 μL lysis mixture (Diacurate), 191 μL water (or 131 μL for whole blood sample), 5 μL probe mix of capture probe and detection probe for Plasmodium sp. (Diacurate), and 6 μL proteinase K (50 g/L) at 56 °C for 30 min with vigorous shaking. Pooled blood spots were lysed in the same manner. The lysates were then transferred, 100 μL per well, to a 96-well capture plate (Diacurate). After overnight incubation at 55 °C, each well was washed 3 times with 150 μL wash buffer (Diacurate) and incubated with 50 μL ligation mix (Diacurate) at 37 °C for 30 min. The plate was then washed again and used for qPCR with 25 μL/well of PCR mixture containing 1× SYBR® Premix Ex (Takara) and 100 nmol/L primers. We performed amplification and detection on a Roche LC480II under the following conditions: 30 s at 95 °C, 45 cycles of 5 s at 95 °C, and 20 s at 60 °C. The melting curve was performed from 65 °C to 90 °C at the default setting.

We prepared standard controls using P. falciparum 3D7 ring-stage synchronized cultured parasites. To measure the percentage parasitemia of the ring stage, an expert microscopist enumerated the infected red blood cells (RBCs) in relation to the number of uninfected RBCs. To determine the number of parasites per microliter in culture material, we multiplied the percent parasitemia by the number of RBCs per microliter counted by the expert microscopist. The standard curve was made by 3-fold serial dilutions from 70 to 0.0012 parasites/μL (11 points) with parasite-negative whole blood lysate. For clinical samples, the results of all positive or discrepant samples, as well as a random sampling of negative samples, were validated with 18S rRNA gene–based Taqman qPCR as previously described (9), for which DNA was...
extracted from 200 μL thawed blood or 10-mm-diameter DBS punch with the QIAamp DNA Blood Minikit (Qiagen) according to the manufacturer’s instructions.

We used Roche LightCycler 480 Software (release 1.5.0) to evaluate the amplification and melting curves and determine the quantification cycle (Cq) values. All experimental samples in this study were tested in duplicate unless noted otherwise. For CLIP-PCR, the sample was considered positive if the fluorescent signal increased within 40 cycles (Cq < 40) and the melting curve was the same as that of the positive control. To generate standard curves, Cq values from duplicate tests were plotted against concentration, and a correlation coefficient was calculated in Microsoft Excel. For standard qPCR, a sample assay cost, we evaluated the ability of CLIP-PCR to detect target RNA in pooled DBSs. Pooling of positive DBS with negative ones did not significantly reduce detection signal (Fig. 3), but too large a pool made it difficult to pipette enough lysate for duplicate tests. A second elution of large pools gave only reduced signal (Fig. 3). Although centrifugation retrieved lysate more effectively, it also added complexity that may add risk for cross-contamination and was not adopted. A pool size of ≤26 was therefore identified as appropriate. To determine the DBS target stability, we spotted cultured P. falciparum (50 parasites/μL) in 75-μL aliquots onto Whatman filter paper and tested after storage with desiccant at room temperature, 4 °C, or −20 °C for 3, 6, 12, and 21 days. We found no significant difference in results for 4 °C or −20 °C storage throughout the experiment. For room temperature storage, there was no significant difference up to 12 days, but at day 21, the RNA quantity was reduced to 55% of the original (data not shown). Therefore, for this assay, the DBS sample could be safely transported with desiccants at room temperature within 12 days with no adverse effect.

For the active screening in this study, we adopted a matrix pooling strategy (Fig. 1). All samples were distributed randomly in a set of $M \times N$ matrix ($M = N$ or $M = N + 1$); each sample was pooled with samples in the same row, and separately with samples in the same column. Pooled samples were lysed and tested as 1 sample by CLIP-PCR. In this way, each sample was tested once in a row pool and once in a column pool. Samples at the intersection of positive row and column pools was tested again as individual confirmation, whereas others were declared negative. In this study, DBS samples arrived in batches. To provide timely diagnosis, samples were tested upon arrival once an adequate pool size of 15–20 was reached.

**Active Screening for Malaria**

With the matrix pooling strategy, we used CLIP-PCR to screen all 3358 DBSs with <500 tests (each test run in duplicate), identifying 14 infections (Table 1). Four infections were found from the cohort of 505 asymptomatic, microscopy-negative people in Gengma County. RDTs were also used for that cohort, and 7 discrepancies with CLIP-PCR were found, all from primary schoolchildren. One of the RDT positives had malaria-related symptoms 1 month before. The corresponding whole blood of discordant samples was tested by standard Taqman qPCR, with all results in favor of CLIP-PCR (Table 1). Follow-up visits 3 months later and reviews of medical history revealed that 1 of the CLIP-PCR positive children developed malaria-related symptoms 10 days after sampling and was confirmed as having P. vivax infection by the local center for disease control and prevention. The remaining 3 CLIP-PCR–positive children developed malaria-related symptoms within 2 months after the sampling day (Table 1). Because of limited local medical

**Results**

**Development of CLIP-PCR**

In CLIP-PCR, quantification of 18S rRNA was achieved by qPCR determination of ligated detection probes that formed only in the presence of the target RNA (Fig. 1). The limit of detection (LOD) was determined as the minimal amount of 3D7 culture added to negative blood lysate that produced a positive result. The LOD was determined to be 0.01 parasites/μL (Fig. 2) for the 3D7 parasite strain.

To increase the assay throughput and reduce per-sample assay cost, we evaluated the ability of CLIP-PCR to detect target RNA in pooled DBSs. Pooling of positive
resources, these individuals turned to private health care providers for treatment without any malaria tests performed. For the other 2 cohorts, CLIP-PCR screening finished independently before the microscopy examinations of the smears were completed. Ten infections were detected among DBS samples collected from the cohort in Tengchong County, all of which were febrile migrant workers with positive microscopy (Table 1). These 10 positive results were also confirmed by standard qPCR: 7 proved to be *P. vivax* infections, 2 were *P. falciparum* infections, and 1 was a mixed infection. The rest of the Tengchong cohort were all negative by CLIP-PCR and microscopy. CLIP-PCR and microscopy found no infections from the cohort in Feidong County. In summary, all positive results from this study were from high-risk populations: 4 from schoolchildren and 10 from a mobile

Fig. 1. Work flow of CLIP-PCR.

CLIP-PCR includes 3 steps. First, sample processing: DBSs are pooled and lysed in 1 step to release 18S rRNA. For pooling, samples in the same row are pooled and tested together as one, and so are the samples in the same column. The schematic gives an example of a 10-by-10 matrix in which sample C5 is tested once in the tube containing pool C and once in the tube containing pool 5. All 100 samples are analyzed twice just by testing pools A–J and pools 1–10 (in a total of 20 tests). Second, formation of PCR template: during overnight incubation of sample lysate, capture probes (CPs) (which includes 2 regions, one for target binding and the other for anchoring the target) and detection probes (DPS) bind to a contiguous part of highly conserved region in *Plasmodium* 18S rRNA; CP anchors the target to solid surface by hybridizing with probes on the solid surface. After the unbound probes are washed off, DPs, which bind adjacent to each other, are ligated to form a longer ssDNA. The detection probes located at both ends also include an extra region as universal primer binding site. Third, quantification: the newly ligated ssDNA, whose quantity is proportional to target RNA, is quantified by qPCR with a universal primer set and SYBR green chemistry.
population returning from malaria-endemic areas. All local adult populations tested negative by CLIP-PCR. To ensure that CLIP-PCR negative results were indeed negative, we randomly picked 44 DBS samples with negative CLIP-PCR and tested with standard qPCR. All proved negative by standard qPCR. To ensure that pooling did not result in any false negatives, 3 asymptomatic clinical DBS samples were separately pooled with 13, 14, and 15 negative samples, and both pooled and individual patient samples were tested by CLIP-PCR. Mean (SD) Cq values of individual samples were 31.02 (0.02), 34.08 (0.67), and 35.07 (0.81), whereas the respective pools had Cq values of 31.52 (0.02), 34.75 (0.41), and 34.51 (0.05). The results confirmed that the pooling strategy minimally compromised the sensitivity of CLIP-PCR.

Discussion

Here we report the development of a novel technology, CLIP-PCR, which retains the high sensitivity of molecular tests while overcoming their most common difficulties. In fact, the analytical LOD of CLIP-PCR reached beyond the ultimate sensitivity limit set by the small sampling volume of the assay. In real settings, the assay has a 20-μL limit of sampling volume for whole blood or equivalent DBS, which would allow only an LOD of 0.05 parasites/μL unless a sample-condensing strategy was considered (15). For DBS samples with the pooling strategy described here, sampling limit sets the LOD to be approximately 0.3 parasites/μL, which is still sufficient to detect subpatent infections.

CLIP-PCR is highly efficient when screening large numbers of samples, since pooled samples are tested in 96-well plates in parallel. In this study, we actively screened 3358 DBS field samples from elimination settings, with a total of <500 tests run in duplicate. We identified 4 asymptomatic infections in addition to 10 microscope-positive results. Three RDT false positives were also identified, demonstrating the high specificity of our assay. The assay is easy to perform, with an ELISA-like workflow that is amenable to automation. Contamination is minimized for CLIP-PCR, and was not found in this study, since both the target and the PCR template are anchored to the bottom of the plate until the closed-well qPCR process starts, and the whole plate is disposed of without opening after PCR. We trained 2 clinical practitioners with little experience of molecular diagnostics for 5 days; these practitioners were able to independently carry out pooling and CLIP-PCR with quality certification. Thus the assay could be deployable in qualified clinical laboratories at the district/county level.
Several characteristics of CLIP-PCR make it highly suitable for large-scale malaria screening in elimination settings. Detecting the much more abundant 18S ribosomal RNA, instead of the DNA, helps to achieve high sensitivity, enabling asymptomatic, subpatent detection. Similar results have been reported by use of standard quantitative reverse-transcription PCR (16). By-passing nucleic acid extraction and reverse transcription with a plate-based sandwich hybridization and target-dependent ligation is the key innovation that drastically improves the throughput of this molecular assay to hundreds of tests per run. Without RNA extraction, assay complexity and cost are reduced.

A dramatic improvement was realized by sample pooling. We showed that pooling of 20 samples before testing further reduced the cost per sample to fractions of a test without pooling, while improving assay throughput to thousands of samples per run, with little compromise of performance (Fig. 3). Unlike earlier tests of malaria with pooling strategy requiring nucleic acid extraction (17, 18), CLIP-PCR sacrifices no apparent sensitivity with the pooling of DBS; Cq values remained almost the same with pool sizes of up to 36. This is not surprising, since pooling of negative DBS dose not dilute the concentration of positive ones in the lysate, and during the overnight incubation, the sandwich hybridization retains with high specificity only target RNA on the surface via cooperative hybridization (19, 20). Nontarget nucleic acids are washed off before formation of the PCR template. In this way, negative samples in each pool have little influence on positive ones. The matrix pooling strategy we adopted ensures that each sample is assayed in duplicate at least twice, with positive ones having a third, individual confirmation run. The matrix testing strategy also helps identify potential sample cross-contamination should it occur. This strategy is suitable for the screening of a large number of people looking for very few infected individuals, since the fewer positives in the sample set, the more savings in the number of tests one has to perform to identify the positives, compared with testing without pooling (21).

CLIP-PCR has several advantages over commonly used methods for DBS samples such as Chelex extraction followed by qPCR. Whereas CLIP-PCR involves only
incubation to elute the target from DBS, the Chelex extraction protocol involves repeated washing in 1 mL PBS and multiple centrifugation and pipetting steps to clean the DBS before elution, which is difficult to perform in regular 96-well plates. The Chelex method is less likely to retain its sensitivity when testing pooled samples, since Chelex extraction does not remove increased level of inhibitors (such as heme) and background DNA that can be detrimental to downstream processes. Therefore, CLIP-PCR is much more suitable for high-throughput processing of pooled DBS and is less expensive on a per-sample basis.

We believe that CLIP-PCR could open a brand new possibility of massive active screening of tens of thousands of people per survey. In such a setting, it will be impractical to perform one-by-one, rapid, field-friendly diagnostic tests, since the logistics of recruiting a large number of individuals for participation is overwhelming. An outsourcing model involving centralized, highly parallel testing in an adequately trained, quality-assured laboratory will make more sense, because it saves resources and time while ensuring high-quality results in a timely fashion. Should subsequent tracking of identified positives become a problem, a focused, much smaller-scale secondary screening can be performed by use of sensitive point-of-care tests such as loop-mediated isothermal amplification (14).

CLIP-PCR can be implemented within the current active surveillance infrastructure as follows. First, DBSs from the entire at-risk community in a village or a border entrance are collected by local disease-control teams and sent to a central clinical laboratory by mail or dedicated carriers. By use of CLIP-PCR with pooling strategy, the laboratory then tests the samples in 96-well plate format with a streamlined, potentially automated workflow. Our experiences suggest that 1 proficient technician can easily test samples from thousands of individuals in a single run. Because the probes target the most conservative region of the *Plasmodium* 18S rRNA, CLIP-PCR should be able to detect all 5 malaria-causing species. Positive samples will be identified within 2–3 days, and an additional day may be used for species identification by use of standard qPCR. The results are quickly fed back to the local and state monitoring agencies via networked rapid communications, followed by rapid response measures such as targeted mass drug administration (3, 4).

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>CLIP-PCR</th>
<th>Cq&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Undetectable</td>
<td>31.0 (0.02)</td>
</tr>
<tr>
<td>61</td>
<td>Undetectable</td>
<td>31.1 (0.17)</td>
</tr>
<tr>
<td>67</td>
<td>Undetectable</td>
<td>31.1 (0.28)</td>
</tr>
<tr>
<td>69</td>
<td>33.9 (1.02)</td>
<td>None&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>80</td>
<td>35.1 (0.81)</td>
<td>None&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>117</td>
<td>34.1 (0.67)</td>
<td>None&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>208</td>
<td>24.4 (0.20)</td>
<td>Fever</td>
</tr>
<tr>
<td>LD572</td>
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<td>Fever</td>
</tr>
<tr>
<td>LD519</td>
<td>28.4 (0.12)</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>Fever</td>
</tr>
<tr>
<td>LD649</td>
<td>21.3 (0.28)</td>
<td>Fever</td>
</tr>
<tr>
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</tr>
<tr>
<td>LD652</td>
<td>21.6 (0.44)</td>
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</tr>
<tr>
<td>2-210</td>
<td>30.0 (0.00)</td>
<td>Fever</td>
</tr>
<tr>
<td>2-8</td>
<td>26.7 (0.05)</td>
<td>Fever</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean (SD) based on duplicate tests.
<sup>b</sup> Whole blood sample.
<sup>c</sup> Patient developed malaria-related syndrome within the following time after sampling: 2 months, 10 days, 1 month, and 2 weeks.
<sup>d</sup> DBS.
<sup>e</sup> NA, not tested.
prevent any onward transmission. The targeted total turnaround time can be less than a week.

With drastically improved throughput, sensitivity, and affordability compared with current screening technology, CLIP-PCR can lead to a higher frequency of active surveys, a larger radius and coverage per survey, and a better detection rate for asymptomatic infections, all without substantial additional expenditures of human and financial resources in current malaria budgets. The major roadblock will shift from technical challenges of detecting all infections in an active survey to operational issues such as how to ensure full participation of a community.

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References