JCM
Journals.ASM.org

Development of a Capillary Electrophoresis-Based Heteroduplex Tracking Assay To Measure In-Host Genetic Diversity of Initial and Recurrent *Plasmodium vivax* Infections in Cambodia

Matthew B. Givens,[a] Jessica T. Lin,[a] Chanthap Lon,[b] Panita Gosi,[b] Meng Chuor Char,[c] Charlotte A. Lanteri,[b] David L. Saunders,[b] Jonathan J. Juliano[a]

Division of Infectious Diseases, University of North Carolina School of Medicine, Chapel Hill, North Carolina, USA[a]; Department of Immunology and Medicine, USAMC Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand[b]; National Center for Parasitology, Entomology, and Malaria Control, Phnom Penh, Cambodia[c]

A heteroduplex tracking assay used to genotype *Plasmodium vivax* merozoite surface protein 1 was adapted to a capillary electrophoresis format, obviating the need for radiolabeled probes and allowing its use in settings where malaria is endemic. This new assay achieved good allelic discrimination and detected high multiplicities of infection in 63 *P. vivax* infections in Cambodia. More than half of the recurrent parasitemias sampled displayed identical or highly related genotypes compared to the initial genotype, suggesting that they represented relapses.

A growing appreciation of the global burden of *Plasmodium vivax* has placed a new emphasis on the development of malaria control and elimination strategies specifically targeting this species (1–3). Molecular tools that sensitively detect minority parasite populations can help achieve a better understanding of *P. vivax* transmission dynamics, the development of drug resistance, and mechanisms underlying hypnozoite relapse (4–7).

Heteroduplex tracking assays (HTAs) are a genotyping method that detects multiple genetic variants coexisting within a single isolate by exploiting the conformational structure of DNA duplexes formed between sample-derived PCR products of a genetic marker of interest and a probe targeting the same marker. Probe-amplicon heteroduplexes each adopt a unique structure based on sequence differences in the sample-derived amplicons, allowing the detection of both single nucleotide polymorphisms and indels. The heteroduplexes migrate differently in a nondenaturing polyacrylamide gel, creating a distinct banding pattern.

We previously used an HTA based on the *P. vivax* merozoite surface protein 1 (*pvmsp1*) gene to show that *P. vivax* infections in Thailand and Cambodia are frequently polyclonal and that some genetic variants may be more prone to relapse than others (6, 8). However, the HTA used a radiolabeled probe, which prevents the assay from being readily adopted in settings where malaria is endemic. To develop a nonradioactive HTA, we adapted the assay to a capillary electrophoresis (CE) format by creating a fluorescently tagged probe and using a nondenaturing polymer.

Fluorescently labeled HTA probes were generated by using previously created plasmids containing 500 bp of variable sequence within the *pvmsp1* gene spanning interspecies conserved blocks 5 and 6, which had been cloned from *P. vivax* strains found in the MR4 repository (MRA-340G, MRA-341G, and MRA-343G) and from a Thai clinical sample (Th06) (8). This approach allows probes to be selected from a single colony, ensuring that they are clonal. Probes were generated by PCR targeting plasmid sections surrounding the insert and labeled on a single strand with a 6-carboxyfluorescein (FAM)-labeled forward primer. For each PCR, 5 μl of 0.2 ng/μl of plasmid was added to 45 μl of a master mixture containing 10× PCR buffer, 200 μM each deoxynucleotide triphosphate (dNTP), 400 nM each primer listed in Table 1, and 1.25 U of HotStarTaq DNA polymerase (Qiagen, Valencia, CA) under the cycling conditions shown in Table 1. The PCR product was run on a 1% agarose gel and purified with the QIAquick Gel Extraction kit (Qiagen, Valencia, CA).

Of the four fluorescently labeled HTA probes generated, the Th06 probe produced the most discrete peak in CE and was chosen for development. We verified that this probe could be used to detect a range of genetic variants by annealing it to *pvmsp1* amplicons from the aforementioned MR4 isolates and finding distinct peaks for each probe-*pvmsp1* amplicon pair by CE. Known mixtures of the *pvmsp1* amplicons from these monoclonal isolates were also created to verify that multiple variants could be detected simultaneously.

The CE-based HTA (CE-HTA) was then applied to samples collected during a malaria cohort study conducted in Oddar Meanchay, Cambodia, in 2010 and 2011 (C. Lon et al., submitted for publication). Genomic DNA was extracted from filter paper blood spots from 64 *P. vivax*-infected persons with the QIAamp 96 DNA Blood kit (Qiagen, Valencia, CA) and amplified in duplicate, with appropriate controls, by nested PCR (Table 1). In the first-round reaction, 2.5 μl of genomic DNA was added to 22.5 μl of a master mixture containing 10× PCR buffer, 200 μM each dNTP, 800 nM each primer, and 0.625 U of HotStarTaq DNA polymerase. In the second-round PCR, 2 μl of the PCR product from round 1 was added to 48 μl of a master mixture containing 10× PCR buffer, 200 μM each dNTP, 800 nM each primer, and 0.625 U of HotStarTaq DNA polymerase. PCR-purified amplicons were annealed to the probe with 1 μl of 1× EDTA buffer, 0.5 μl of 20 μM *pvmsp1* HTA F primer, 4.5 μl of 5 μM probe, and 4 μl

Received 19 August 2013 **Returned for modification** 5 September 2013
Accepted 8 October 2013
Published ahead of print 16 October 2013
Editor: M. J. Loeffelholz
Address correspondence to Jessica Lin, jessica_lin@med.unc.edu.

doi:10.1128/JCM.02274-13

TABLE 1 Sequences of the primers and probes used in the HTA

Amplification	Primer sequence	Amplification program
pvmsp1 probe		
HTA forward	5′-FAM-TGCAGGTCGACTCTAGAGGAT-3′	95°C for 15 min; 20 cycles of 95°C for 15 min, 55°C for 2 min, 72°C for 1 min; 72°C for 10 min
HTA reverse	5′-ACGGCCAGTGAATTCGAG-3′	
pvmsp1 ICB5-6 amplicon		
Round 1		
MB5W	5′-AGACARGCGAGAAACCTG-3′	94°C for 10 min; 35 cycles of 94°C for 50 s, 50°C for 50 s, 72°C for 1 min; 72°C for 2 min
MB6	5′-TATATATTGTAAACCATTTCC-3′	
Round 2		
pvmsp1 HTA F	5′-GATGGTCCTCAAAAGGGAAA-3′	94°C for 10 min; 35 cycles of 94°C for 50 s, 55°C for 50 min, 72°C for 1 min; 72°C for 2 min
pvmsp1 HTA R	5′-GACATGCGTAAGCGGATTTT-3′	

of PCR-purified *pvmsp1* amplicon. The mixture was heated to 95°C for 2 min and then cooled to 25°C for 5 min. A 1.5-μl volume of the annealed mixture was then prepared for CE on a 310 Genetic Analyzer (Life Technologies, Carlsbad, CA) by adding 10.5 μl of highly deionized formamide and 0.6 μl of GeneScan 1200 LIZ Size Standard (Life Technologies). CE was carried out with a 61-cm capillary and a diluted polymer consisting of 600 μl of POP Conformational Analysis Polymer (Life Technologies), 263 μl of deionized water, 108 μl of autoclaved glycerol, and 108 μl of 10× EDTA. Duplicate samples were run with the following parameters: a 30°C run temperature, a 9.5-kV injection voltage, a 10-s injection length, a 7-kV run voltage, and a 180-min run length.

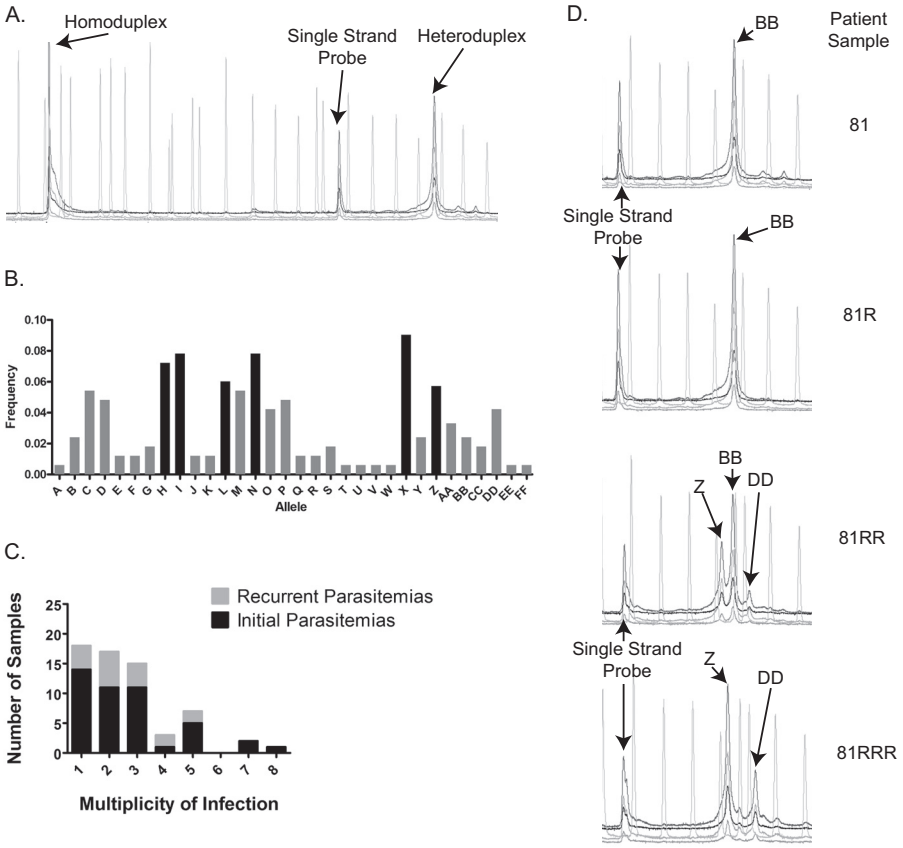

FIG 1 (A) Sample CE-HTA output. Each sample run contained peaks corresponding to the single-stranded probe, probe homoduplex (double-stranded probe reannealed to itself), and one or more heteroduplex peaks (probe bound to *pvmsp1* fragment) representing different *pvmsp1* variants. Light gray peaks represent size standards. (B) Allele frequencies of *pvmsp1* sequence variants by HTA migration index. A total of 166 clones were identified from 63 *P. vivax* infections. The six most common clones, which accounted for 41% of the total, are shaded dark gray. (C) Distribution of MOIs. The number of coinfecting *pvmsp1* clones per isolate among the 63 Cambodian samples (45 initial and 18 recurrent) is shown. (D) CE-HTA peaks in a subject with recurrent parasitemias. The relatedness of variants in successive infections is highlighted in subject 81, who suffered three consecutive recurrences, each about 5 weeks following treatment with dihydroartemisinin-piperaquine.

In the end, 63 samples from 45 persons were successfully amplified in duplicate and evaluated by CE-HTA. With the GeneMapper software (Applied Biosystems), CE-HTA peaks with heights of >50 U were assigned a migration index based on their migration relative to the size standard (Fig. 1A). It is important to note that the size standard was used in a nontraditional manner, as a way to quantify relative migration and not base pairs, thus acting as a proxy for migration efficiency. Allele assignments were made by placing the heteroduplex peaks into bins of 10 size standard units, as this was the standard deviation of the migration index for the homoduplex and single-stranded probe peaks among all of the runs.

A total of 32 distinct alleles were identified among the 45 subjects. These were alphabetically labeled A to FF, with the heteroduplex formed by allele A with the probe adopting the most efficient conformation and exhibiting the fastest migration (Fig. 1B). The most frequent allele was allele X, which accounted for 9% (15/166) of the clones identified and was present in nearly a quarter of the samples (15/63, 24%). The six most common alleles accounted for 44% of the clones identified. Impressively, the virtual heterozygosity (H_E), a measurement of the genetic diversity of a given locus within the study population, was calculated to be 0.95. This was greater than the H_E of 0.86 we previously found with the traditional HTA format, likely because of the higher discriminatory power afforded by the CE format. This H_E is comparable to the H_E of the most discriminatory microsatellite markers used to genotype *P. vivax* (8, 9). Unfortunately, the *pvsmp1* CE-HTA probe selected was based on a slightly different *pvmsp1* sequence, precluding a direct comparison with our prior study.

In addition to showing more allelic discrimination than the traditional HTA, the CE-HTA maintained sensitive detection of minority variants within isolates. For each sample run, the number of distinct heteroduplex peaks in separate bins was summed as the sample's multiplicity of infection (MOI). Only peaks that were duplicated between both replicates of a given sample were counted. The mean calculated MOI was 2.6, with a range of 1 to 8 (Fig. 1C). Seventy-one percent of the samples were polyclonal, containing more than one variant. These estimates are consistent with our prior Cambodian study (8) and on a par with MOI estimates from Thailand and Papua New Guinea based on microsatellite markers (10, 11).

As part of the cohort study, subjects who developed *P. vivax* malaria were treated with dihydroartemisinin-piperaquine by directly observed therapy, followed weekly by light microscopy confirmed retrospectively by PCR for at least 42 days posttreatment, and then followed monthly with the same testing until the study ended. They were not treated with primaquine to prevent relapse until the end of the study. Our study sample contained 11 persons who suffered one or more recurrent parasitemias at a median of 63 days posttreatment that could represent relapses (Table 2; Fig. 1D). When consecutive parasitemias in these persons were compared, 80% (12/15) of the initial and recurrent genotypes were "related" in that they had one or more *pvmsp1* variants in common. Two of these pairs (2/12) contained identical genotypes, while two-thirds (8/12) were "highly related" in that at least half of the variants seen in the recurrent parasitemia were also seen in the initial or immediately preceding parasitemia. The last two (2/12) were not highly related (81R→81RR and 80→80R). This supports our previous observation that, even with the use of a highly discriminatory assay, the bulk of recurrent *P. vivax* parasitemias in Cambodia appear to be related (8). Still, half of the related pairs (6/12) contained a novel variant in the recurrent parasitemia.

TABLE 2 Genotypes of subjects with recurrent *P. vivax* parasitemia(s) by HTA

Sample	MOI	Variant(s)	Time to recurrence (days)
Identical and identical-related pairs			
81	1	BB	
81R[a]	1	BB	35
81RR	3	Z BB DD	34
81RRR[a]	2	Z DD	31
151	2	C D	
151R[a]	2	C D	126
Related pairs			
80	7	D E H I N O FF	
80R	5	B I Y Z BB	56
80RR[a]	4	B L Z CC	42
87	3	M Z AA	
87R[a]	2	B M	81
96	2	M AA	
96R[a]	1	AA	71
123	1	X	
123R[a]	2	C X	26
130	5	N P S T X	
130R[a]	3	P X DD	68
130RR[a]	2	P X	43
153	5	H K O S X	
153R[a]	1	X	115
Unrelated pairs			
125	1	Y	
125R	3	H I R	82
152	4	G H I L	
152R	1	X	94
154R	2	H J	64
154RR	4	I K L N	62

[a] Identical or highly related recurrent parasitemias that are suspected to arise from relapse.

We believe the 10 identical or highly related genotype pairs are more likely to represent relapse rather than reinfection (indicated by asterisks in Table 2). Recrudescence of the same alleles because of treatment failure after dihydroartemisinin-piperaquine is felt to be unlikely. Five novel variants were seen in 4 of these 10 recurrences suspected to represent relapses. The novel variants seen in these suspected relapses (often referred to as heterologous hypnozoites [12]) may represent latent hypnozoites inoculated more remotely prior to the initial parasitemia or minority variants not readily detectable at the time of initial sampling. However, another possibility is that reinfection also triggers the relapse of latent hypnozoites. It is important to note that the initial parasitemias may themselves also represent relapses from infection prior to study enrollment.

In conclusion, we have successfully combined the sensitive detection of minority variants by HTA with the practical advantages of using CE for malaria genotyping. The *pvmsp1* CE-HTA reported here achieved a high level of allelic discrimination among *P. vivax* parasites in a northern Cambodian cohort while revealing the relatedness of parasites found in initial and recurrent episodes. The probe for this CE-HTA is nonradioactive and easily gener-

ated, as once a gene target sequence is cloned, it takes only a simple PCR using universal primers to create a fluorescently labeled probe; however, probe sequences should be standardized for comparison between genotyping studies. This practical method of characterizing in-host diversity in clinical *P. vivax* infections can be used to shed light on *P. vivax* transmission and mechanisms of relapse, an understanding of which is important to guide future malaria elimination strategies in areas where *P. vivax* is endemic.

Nucleotide sequence accession number. The sequence obtained in this study has been deposited in GenBank under accession number KF509888.

ACKNOWLEDGMENTS

We thank Oksana Kharabora and Jaymin Patel for their technical support in the laboratory. We are indebted to the volunteers in the clinical study, as well as the dedicated field staff at AFRIMS and the Cambodia National Malaria Program who carried out the clinical study.

This work was supported by the U.S. Army Medical Materiel Development Activity, Fort Detrick, MD, and the National Institutes of Health (grant AI089819 to J.J.J.). M.B.G. was supported by a University of North Carolina Summer Undergraduate Research Fellowship. J.T.L. was supported by an ASTMH/Burroughs Wellcome Postdoctoral Fellowship in Tropical Infectious Disease and the North Carolina Clinical and Translational Science Award (grant UL1RR025747).

The views expressed in this paper are ours and do not represent the official position of the U.S. Department of Defense.

REFERENCES

1. **Gething PW, Elyazar IRF, Moyes CL, Smith DL, Battle KE, Guerra CA, Patil AP, Tatem AJ, Howes RE, Myers MF, George DB, Horby P, Werheim HF, Price RN, Mueller I, Baird JK, Hay SI.** 2012. A long neglected world malaria map: *Plasmodium vivax* endemicity in 2010. PLoS Negl. Trop. Dis. **6:**e1814. http://dx.doi.org/10.1371/journal.pntd.0001814.
2. **World Health Organization.** 2013. *Plasmodium vivax* control & elimination: development of global strategy and investment case, September 2012. World Health Organization, Geneva, Switzerland. http://www.who.int/malaria/mpac/sep2012/pvivax_development_global_strategy_and_investment_sep2012.pdf. Accessed 4 August 2013.
3. **Shanks GD.** 2012. Control and elimination of *Plasmodium vivax*. Adv. Parasitol. **80:**301–341. http://dx.doi.org/10.1016/B978-0-12-397900-1.00006-2.
4. **de Brito CF, Ferreira MU.** 2011. Molecular markers and genetic diversity of *Plasmodium vivax*. Mem. Inst. Oswaldo Cruz **106:**12–26. http://dx.doi.org/10.1590/S0074-02762011000900003.
5. **Mideo N, Kennedy DA, Carlton JM, Bailey JA, Juliano JJ, Read AF.** 2013. Ahead of the curve: next generation estimators of drug resistance in malaria infections. Trends Parasitol. **29:**321–328. http://dx.doi.org/10.1016/j.pt.2013.05.004.
6. **Lin JT, Juliano JJ, Kharabora O, Sem R, Lin FC, Muth S, Menard D, Wongsrichanalai C, Rogers WO, Meshnick SR.** 2012. Individual *Plasmodium vivax msp1* variants within polyclonal *P. vivax* infections display different propensities for relapse. J. Clin. Microbiol. **50:**1449–1451. http://dx.doi.org/10.1128/JCM.06212-11.
7. **De Araujo FCF, De Rezende AM, Fontes CJF, Carvalho LH, Alves de Brito CF.** 2012. Multiple-clone activation of hypnozoites is the leading cause of relapse in *Plasmodium vivax* infection. PLoS One **7:**e49871. http://dx.doi.org/10.1371/journal.pone.0049871.
8. **Lin JT, Patel JC, Kharabora O, Sattabongkot J, Muth S, Ubalee R, Schuster AL, Rogers WO, Wongsrichanalai C, Juliano JJ.** 2013. *Plasmodium vivax* isolates from Cambodia and Thailand show high genetic complexity and distinct patterns of P. vivax multidrug resistance gene 1 (pvmdr1) polymorphisms. Am. J. Trop. Med. Hyg. **6:**1116–1123. http://dx.doi.org/10.4269/ajtmh.12-0701.
9. **Koepfli C, Mueller I, Marfurt J, Goroti M, Sie A, Oa O, Genton B, Beck HP, Felger I.** 2009. Evaluation of *Plasmodium vivax* genotyping markers for molecular monitoring in clinical trials. J. Infect. Dis. **199:**1074–1080. http://dx.doi.org/10.1086/597303.
10. **Imwong M, Nair S, Pukrittayakamee S, Sudimack D, Williams JT, Mayxay M, Newton PN, Kim JR, Nandy A, Osorio L, Carlton JM, White NJ, Day NP, Anderson TJ.** 2007. Contrasting genetic structure in *Plasmodium vivax* populations from Asia and South America. Int. J. Parasitol. **37:**1013–1022. http://dx.doi.org/10.1016/j.ijpara.2007.02.010.
11. **Koepfli C, Ross A, Kiniboro B, Smith TA, Zimmerman PA, Siba P, Mueller I, Felger I.** 2011. Multiplicity and diversity of *Plasmodium vivax* infections in a highly endemic region in Papua New Guinea. PLoS Negl. Trop. Dis. **5:**e1424. http://dx.doi.org/10.1371/journal.pntd.0001424.
12. **White NJ.** 2011. Determinants of relapse periodicity in *Plasmodium vivax* malaria. Malar. J. **10:**297. http://dx.doi.org/10.1186/1475-2875-10-297.