New developments in malaria diagnostics
Monoclonal antibodies against Plasmodium dihydrofolate reductase-thymidylate synthase, heme detoxification protein and glutamate rich protein

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Abbreviations: RDTs, rapid diagnostic tests; GLURP, glutamate rich protein; DHFR-TS, dihydrofolate reductase-thymidylate synthase; HDP, heme detoxification protein; K_D, dissociation constant; HRP2, histidine rich protein; pLDH, parasite lactate dehydrogenase

Introduction

Accurate early diagnosis and subsequent treatment are essential to reduce morbidity and mortality due to malaria. Diagnosis of malaria based on clinical signs and symptoms may lead to over-diagnosis even in highly endemic areas, as the clinical presentations of the disease resemble those of a number of other common illnesses, such as pneumonia, equally associated with morbidity and mortality.1-3 Introduction of effective but expensive, artemisinin-based combination therapies, due to increasing parasite resistance to older drugs such as chloroquine and sulfadoxine-pyrimethamine, increase the urgency for cheap and accurate diagnosis.4

Microscopic examination of blood slides is, in principle, sensitive and specific for the parasitological detection of malaria, if used correctly, and has been used for over a hundred years. Unfortunately, it is not always widely available in many highly endemic areas or it is poorly executed, and for that reason an easy-to-use alternative is needed in situations where good quality microscopy is unavailable.5

Over a decade ago, rapid diagnostic tests (RDTs) for malaria were introduced that have the advantages of being quick and can be easily performed in remote settings.6,7 These lateral flow immuno-chromatographic tests are based on the detection of malaria parasite antigens in patient blood by specific monoclonal antibodies (mAbs) that bind the antigen and immobilize and mark it with a label on nitrocellulose. Commercially available RDTs for malaria all detect one or more of the following antigens: histidine rich protein 2 (HRP2), lactate dehydrogenase (pLDH) or aldolase. Tests detecting HRP2 are most commonly used because of their lower cost, better stability and lower detection threshold compared with pLDH-based tests.8-11 Moreover,
HRP2-based RDTs have better sensitivity than pLDH-based tests, although specificity is better for pLDH-based tests.12 HRP2-based tests, however, only detect *Plasmodium falciparum* and the genetic diversity within the HRP2 antigen affects RDT sensitivity.13,14 Sensitivities for aldolase-detecting RDTs have improved of less than 15% with *plasmodium HDP*.23 *HDP* is present in several life stages of *Plasmodia* and in the early ring stage it is trafficked to the food vacuole in transport vesicles together with heme.23

Glutamate Rich Protein (GLURP) was chosen because it is a highly antigenic exoantigen of *P. falciparum* that is expressed in all parasite life stages in the host, and its gene is conserved in geographically different isolates.23 The protein includes two repeat regions, R1 and R2, of which the R2 repeat sequence is well conserved between different isolates.24 Additionally, several major B-cell epitopes in the R0 (non-repetitive) region of GLURP have been identified.25 This target is only suitable for *P. falciparum* diagnosis, because the gene is not present in the other species, but it might be used in combination with other antibodies to make a diagnostic test against multiple species.

mAbs were raised against the *P. falciparum* specific antigen glutamate rich protein (GLURP) and the PAN-plasmodial antigens dihydrofolate reductase-thymidylate synthase (DHFR-TS) and heme detoxification protein (HDP). These antibodies were screened for specificity against *P. falciparum* and *Plasmodium vivax*. Subsequently, affinity and detection limit were determined and compared with commercially available HRP2 antibodies.

**Results**

**Generation and screening of mAbs.** Antibody response to the antigens in mouse serum was measured during the immunization process. High titers were observed for all antigens and 20 x 10⁶ to 390 x 10⁶ cells/ml could be harvested from the spleens (Table 1). Half of the cells from one mouse for each antigen with the highest titer or cell count were fused with myeloma cells, except for GLURP R2, where the cells might have been contaminated during harvest of the mouse with the highest titer. After selection of hybridoma cells on selective medium with aminopterin, on average 45% of the wells contained growing clones. These cells were screened for specificity against *P. falciparum* and *Plasmodium vivax*. Subsequently, affinity and detection limit were determined and compared with commercially available HRP2 antibodies.

**Table 1.** Antibody response of mouse sera to the recombinant antigen in ELISA at different days before each (booster) immunization during the immunization procedure

| Antigen     | Mouse | Before immunization (Day 0) | Day 29     | Day 42     | Day 48     | #Cells * 10⁶ harvested/ml |
|-------------|-------|----------------------------|------------|------------|------------|--------------------------|
| GLURP R₁    | A     | 0                          | 1:12,000   | ≥1:15,36,000| 1:3,20,000 | 60                       |
|             | B     | 0                          | 1:24,000   | ≥1:15,36,000| 1:24,000   | 30                       |
| GLURP R₂    | A¹    | 0                          | 1:3,000    | ≥1:15,36,000| 1:12,00,000| 70                       |
|             | B¹    | 0                          | 1:24,000   | ≥1:15,36,000| 1:12,00,000| 1:61,44,000*** 220       |
| DHFR-TS     | A     | 0                          | 1:12,000   | ≥1:15,36,000| 1:3,20,000 | 60                       |
|             | B¹    | 0                          | 1:24,000   | ≥1:15,36,000| 1:24,000   | 70                       |
| DHFR-1&2    | A     | 0                          | 1:12,000   | ≥1:15,36,000| 1:102,40,000| 20                       |
|             | B¹    | 0                          | 1:24,000   | ≥1:15,36,000| 1:102,40,000| 20                       |
| HDP         | A     | 1:3,000                    | 1:12,000*  | 1:48,000** | ≥1:61,44,000*** 220       |
|             | B     | 0                          | 1:24,000*  | 1:96,000** | 1:15,36,000*** 390       |

Mouse A of HDP already had a background titer at Day 0. *This mouse was used for the fusion to myeloma cells; *day 28; **day 35; ***day 42.
the corresponding recombinant antigen (Table 2). For each antigen, the most reactive antibodies were selected and these cell lines were further sub-cloned to derive monoclonal hybridoma cell lines for further characterization. Isotyping of selected clones was performed and, for the DHFR-TS antibodies, mostly IgM antibodies were selected, but for GLURP and HDP a larger proportion of mAbs were IgG (Table 2).

**Dissociation constant of antibodies.** Dissociation constants were estimated by ELISA and are listed in Table 3 for selected DHFR-TS, HDP and GLURP antibodies. The DHFR-TS antibodies with the highest affinities are D5 and D6, for HDP H16 and H18 and for GLURP are G22 and G23 (Table 3). The dissociation constants of the commercially obtained HRP2 antibodies were not found in literature, but were measured in the present study as 0.16 ± 0.13 nM for PTL3 and 1.0 ± 0.049 nM for C1–13. This is in the same order of magnitude as the antibodies against P. falciparum recombinant antigens described by Tomar et al. Many of the developed antibodies for DHFR-TS, HDP and GLURP have a similar or higher affinity for their antigen than the HRP2 antibodies.

**Crude parasite antigen detection.** The ELISA with crude parasite antigen allowed the detection of native DHFR, HDP and GLURP antigens from culture at different concentrations by selected mAbs. The lower detection limit of several developed antibodies was comparable to the detection limit of two HRP2 antibodies (Table 4). The detection of antibody, expressed as absorbance at 450 nm, was linearly correlated with the antigen concentration for all described mAbs (r ≥ 0.93 and p < 0.05) (Fig. 1A–D). The strongest antibodies, D6, D15, H12 and H16 are very comparable in absorbance pattern to the HRP2 antibodies (Fig. 1A).

**Detection of *P. vivax.*** Although the antibodies were raised against *P. falciparum* recombinant antigens, five HDP (H2, H7, H10, H13 and H16) and four DHFR-TS (D7, D15, D16 and D28) of the selected antibodies were able to detect pooled *P. vivax* patient samples in ELISA, besides *P. falciparum*. Positive absorbance at 450 nm in the ELISA ranged from 0.31–1.06 compared with 0.24 ± 0.03 of background absorption (no-antibody negative controls) and 0.22 ± 0.02 of GLURP (Pf specific) negative controls.

### Table 2. Overview and characteristics of hybridoma clones during immunization and selection process

| Antigen target | Nr of wells started with | With recombinant antigen | With NF54 | #selected clones based on ELISA | Proportion IgG | Proportion IgM |
|----------------|-------------------------|--------------------------|-----------|-------------------------------|----------------|----------------|
| DHFR-TS insert 1 and 2 | 1008 | 8% | 9% | 16 | 1/16 | 15/16 |
| TS | 960 | 8% | 6% | 12 | 2/12 | 10/12 |
| GLURP R_5 | 1504 | 5% | 5% | 4 | 1/4 | 3/4 |
| GLURP R_9 | 576 | 9% | 11% | 10 | 7/10 | 3/10 |
| HDP | 2112 | 10% | 5% | 33 | 9/33 | 24/33 |

### Table 3. Dissociation constants ($K_D$) of monoclonal antibodies as determined by ELISA

| Target | mAb | Isotype | $K_D$ (nM) | SE | Target | mAb | Isotype | $K_D$ (nM) | SE | Target | mAb | Isotype | $K_D$ (nM) | SE |
|--------|-----|---------|------|----|--------|-----|---------|------|----|--------|-----|---------|------|----|
| DHFR   | D5  | IgG3    | 0.10 | 0.014 | HDP  | H1  | IgG1    | 1.5  | 0.64 | GLURP | G2  | IgG3    | 1.42 | 0.27 |
| D6     | IgM | 0.068   | 0.015 |      | H2   | IgM    | 0.93 | 0.11 | G4    | IgG1 | 3.4     | 0.45 |
| D7     | IgM | 0.36    | 0.039 |      | H3   | IgM    | 0.23 | 0.046 | G12   | IgM  | 0.54    | 0.089 |
| D10    | IgM | 0.66    | 0.19  |      | H4   | IgG2b  | 0.23 | 0.057 | G13   | IgM  | 0.45    | 0.079 |
| D13    | IgM | 0.41    | 0.20  |      | H5   | IgG3   | 2.1  | 0.82 | G14   | IgG2a| 0.53    | 0.64 |
| D15    | IgM | 0.80    | 0.49  |      | H6   | IgM    | 0.70 | 0.16 | G15   | IgG2a| 0.54    | 0.11 |
| D16    | IgM | 0.16    | 0.062 |      | H7   | IgM    | 0.32 | 0.030 | G16   | IgM  | 0.94    | 0.26 |
| D19    | IgG1| 0.88    | 0.32  |      | H8   | IgM    | 0.56 | 0.081 | G18   | IgG1 | 0.70    | 0.12 |
| D20    | IgM | 0.21    | 0.10  |      | H12  | IgG2a  | 0.83 | 0.25 | G19   | IgG3 | 0.56    | 0.10 |
| D24    | IgM | 2.4     | 1.3   |      | H16  | IgM    | 0.10 | 0.022 | G22   | IgG1 | 0.33    | 0.093 |
| HRP2   | PTL3| IgM     | 0.16  | 0.13  | H18  | IgM    | 0.21 | 0.022 | G23   | IgM  | 0.11    | 0.028 |
| C1-13  | IgG | 1.0     | 0.049 |      | G24  | IgM    | 0.34 | 0.14 | G25   | IgM  | 1.4     | 0.33 |

**Discussion**

Concerns regarding the diagnostic accuracy and test stability of malaria RDTs have prompted development of novel antibodies that could be incorporated in new diagnostic tests. In the present study, novel mAbs were developed and characterized against the newly selected Plasmodium antigen targets dihydrofolate reductase-thymidylate synthase, heme detoxification protein and glutamate rich protein.
The present work generated several mAb-producing hybridoma cell lines, and specific clones were selected on the basis of their reactivity with recombinant antigen and malaria cell culture material. Affinities of the developed antibodies for their recombinant antigens are higher than, or comparable to, the two tested commercially available HRP2 antibodies. High affinity is important for sensitivity and the detection limit of diagnostic assays, suggesting that the newly developed antibodies are good candidates for diagnostic test development. The antibodies can detect the native antigens from NF54 and 3D7 because the antibody affinities are very similar. The abundance of the antigen in clinical samples is important for the detection limit of a diagnostic assay, and should be kept into consideration in further test development. Some of the antibodies could also detect Plasmodium malariae and Plasmodium ovale parasites. The mAbs presented in this study show much potential for further development in diagnostic assays.

### Material and Methods

#### In vitro culture of *P. falciparum*

Plasmodium falciparum strains NF54 (UMC St. Radboud, Nijmegen, The Netherlands) and 3D7 (MRA-102, MR4, ATCC® Manassas Virginia) parasites were cultured in a candle jar on RPMI 1640 medium with L-Glutamine and 25 mM HEPES (Gibco, Invitrogen) with human A+ serum and O+ red blood cells according to the candle-jar technique of Trager-Jensen.28,29 The parasitemia was determined with Fields-stained microscopy slides and counted as percentage of infected red blood cells. The parasite strain used in the experiments depends on the isolate that was in culture because the department changed the parasite strain to be used as a standard from NF54 to 3D7. NF54 is the parental isolate of clone 3D7; therefore no difference is expected between the clones concerning these antigens. The recombinant antigens were amplified from NF54 parasites (see section below) and the vectors were sequenced and compared with the published 3D7 genes in PlasmoDB; the sequences were identical (data not shown).30

#### Preparation of *P. falciparum* lysate

Parasite culture (~2–5%) was diluted 10 times in PBS and sonicated vigorously for 30 sec. This suspension was used in the ELISA procedures described below.

#### *P. vivax* samples

Frozen samples from patients infected with *P. vivax* parasites were provided by the Instituto de Medicina Tropical “Alexander von Humboldt”—Universidad Peruana Cayetano de Heredia in Peru. Samples were characterized with microscopy. A pool of samples was used with a resulting parasitemia of ~0.1% and used in the screening ELISA described below.

#### Production of recombinant antigens

The recombinant HDP protein was provided for the purpose of this study from the Virginia Bioinformatics Institute, Virginia Tech.23 Primers were developed to amplify gene fragments of the DHFR-TS and GLURP antigens and sequences are listed in Table 5. Genetic sequences were derived from PlasmoDB, with the following accession numbers: DHFR-TS: PFD0830w and GLURP: PF10_0344.30 Amplification was performed with Accuprime Taq High Fidelity (Invitrogen) and the supplied PCR buffer II with the addition of 0.05 mM dNTPs and 0.2 μM each of forward and reverse primer. Amplification was performed on a Peltier Thermal cycler 100 with initial hot start of 1 min at 94°C and subsequently 40 cycles of 30 sec at 94°C, 30 sec at 58°C.

### Table 4. Detection limit of crude parasite antigen in ELISA by selected antibodies compared with HRP2 antibodies

| Antigen | mAb | Detection limit of crude antigen |
|---------|-----|---------------------------------|
| HRP2    | C1-13 | 250 | 8 |
| HRP2    | C1-13 | 25 | 8 |
| DHFR    | D6   | 250 | 25 |
| DHFR    | D15  | 25 | 25 |
| HDP     | H4   | 25 | 8 |
| HDP     | H12  | 25 | 8 |
| HDP     | H12  | 250 | 3 |
| HDP     | H15  | 250 | 3 |
| HDP     | H16  | 250 | 25 |
| HRP2    | PTL3 | 25 | 8 |
| HRP2    | PTL3 | 250 | 3 |
E. coli High Efficiency (New England Biolabs) with heat shock at 42°C for 10 sec. Recombinant antigens were expressed and purified with Chitine beads as described in the IMPACT KIT (New England Biolabs) and confirmed on SDS-PAGE gel. This work was performed in a controlled environment under GMO license nr 02–080 of the Dutch ministry of Infrastructure and Environment.

Immunization protocol and hybridoma culture. Ten to twelve week old specific pathogen free Balb/c mice (two for...
each antigen) were immunized twice (with a 28-d interval) with 100 μg recombinant antigen in PBS with 50% Stimune adjuvant (Prionics) intra peritoneal (i.p.) and subcutaneous (s.c.) and with a final booster after 11 d with 100 μg recombinant antigen in PBS intravenous (i.v.). All animal experiments were approved by the local animal ethics committee. Antibody response in mice serum was determined with ELISA as described below. Splenocytes were harvested and frozen according to the protocol from Marusich et al. The cells of half a spleen were fused to myeloma cells according to a standardized protocol. Cell lines were selected based on ELISA results described below, and subsequently sub-cloned to obtain single clones. Hybridoma cells were grown in Optitrem I with Glutamax (Invitrogen) with 10% FCS (Fetalclone I, Hyclone) or Hybridoma Serum Free medium (Invitrogen).

**Screening ELISA.** Mice serum and supernatant of hybridoma cell lines were screened with ELISA for specific reactivity with recombinant antigen and NF54 and/or 3D7 culture lysate. ELISA plates (medium binding, Greiner) were coated over night at 4°C with recombinant antigen (20 μg/ml) in PBS or P. falciparum lysate or a P. vivax patient sample (5,630 parasites/μl diluted 1:5 in PBS) and subsequently washed with PBS + 0.1% Tween 20 (PBST) and blocked with 5% BSA (Sigma) in PBS for 1 h at 37°C. Plates were washed and incubated 1 h at 37°C with 50 μl of supernatant of the hybridoma clones or mouse serum diluted in 1% BSA in PBST (1:3 x 10^3 –1:10 x 10^6 ). After washing, plates were incubated 1 h at 37°C with peroxidase conjugated goat-anti-mouse (Jackson Immunoresearch) 1:5,000 diluted in 1% BSA in PBST. The presence of antibody was measured by adding 0.04% 3,3′,5,5′-Tetramethylbenzidine (Sigma), 0.04% urea peroxide (Sigma) in 0.1 M Sodium acetate citrate pH 4 and the reaction was stopped with 0.5 M Sulfuric acid. Color development was measured at 450 nm in a SUNRISE ELISA reader (Tecan).

**Purification of mAbs.** Antibodies were isotyped (Pierce Rapid ELISA Mouse Antibody Isotyping Kit, Thermo Scientific) and HDP and DHFR-TS antibodies were purified directly from hybridoma supernatant (filtered through 0.2 μm) with 1 ml HiTrap protein G HP columns (GE Healthcare) in case of IgG and with 1 ml HiTrap IgM purification HP columns (GE Healthcare) in case of IgM, according to manufacturer’s instructions. Purifications were performed on an ÄKTA FPLC system (Amersham Biosciences). Antibody containing fractions were pooled and concentrated with 50 kD (IgG) or 100 kD (IgM) Amicon® Ultra-4 Centrifugal Filter Units (Millipore) until a concentration of approximately 1 mg/ml was reached. Antibody concentration was determined by adsorption at 280 nm with a Nanodrop 1000 (Thermo Scientific).

The GLURP antibodies used in the experiments were precipitated with ammonium sulfate. Hybridoma supernatant was clarified by a 0.2 μm syringe filter (Millipore) and saturated ammonium sulfate pH 7.4 was slowly added till the final concentration of ammonium sulfate was 45% and left on a rotator for 45 min. Subsequently, the mixture was incubated O/N at 4°C and then centrifuged at 3,893 g for 40 min. The pellet was resuspended in 8 ml cold PBS and concentrated with 50 kD Amicon® Ultra-4 Centrifugal Filter Units (Millipore) until a concentration of approximately 1 mg/ml was reached.

**Measurement of true affinity (K_{D}) by ELISA.** The binding affinities of the antibodies were determined by measuring the dissociation constant (K_{D}). Briefly, antigen at various concentrations ranging from 1 x 10^{-7} M to 5 x 10^{-11} M was mixed with a constant amount of antibody in 0.1 M potassium phosphate, 2 mM EDTA, 10 mg/ml BSA, pH 7.8 and incubated for 15 h at room temperature. The antigen-antibody mix was then transferred to micro-titer plates previously coated with capture antigens at 150 ng/well in 50 mM sodium carbonate pH 9.6 (DHFR1&2, DHFR-TS, GLURP R, GLURP R or HDP) and incubated for 60 min at room temperature. Washing of plates and detection of the presence of antibody was performed as described for the screening ELISA. Dissociation constants were calculated using regression analysis and the Scatchard-Klotz equation.

In comparison, two HRP2 antibodies (C1–13 and PTL3, National Bioproducts Institute) were tested as well with recombinant HRP2 (ReaMatrix).

**Crude parasite antigen ELISA.** A crude parasite antigen extract was prepared from a late stage 3D7 culture (-3–4% schizonts) with Percoll as described by Troye-Blomberg et al. This antigen extract was coated overnight on ELISA plates in a dilution series from 20-0.03μg/ml in 1% BSA in PBST. 1% BSA in PBST was used as negative control and the wells were subsequently blocked with 5% BSA in PBS for 30 min to 1 h at 37°C. After washing 3 times with PBST, purified antibodies (5 μg/ml) were added to each dilution series. In comparison two HRP2 antibodies (C1–13 and PTL3, National Bioproducts Institute) were taken along on each ELISA plate in the same concentration.

**Statistical analysis.** The dissociation constant (K_{D}) was determined by calculating the slope of the lines by regression analysis. The correlation between absorbance in the ELISA and the antigen concentration was determined by Pearson product moment correlation coefficient (PMCC).

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