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Minimising invasiveness in diagnostics: developing a rapid urine-based monoclonal antibody dipstick test for malaria

Uri S. Markakpo[1,2], Kwabena M. Bosompem[2], Mawuli Dzodzomenyo[1], Anthony Danso-Appiah[1], Edward E. Essuman[1], William K. Anyan[2], Mitsuko Suzuki[2,3], Judith K. Stephens[1], Isaac Anim-Baidoo[4], Richard H. Asmah[4], Michael F. Ofori[2], Parnor Madjitey[1], Jonas B. Danquah[1], Naa Adjeley Frempong[2], Kofi D. Kwofie[2], Michael Amoa-Bosompem[2], David Sullivan Jr[5], Julius N. Fobil[1] and Isabella A. Quakyi[1]

1 *School of Public Health, University of Ghana, Legon, Ghana*
2 *Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana*
3 *Section of Environmental Parasitology, Tokyo Medical and Dental University, Tokyo, Japan*
4 *School of Allied Health Sciences, University of Ghana, Korlebu, Ghana*
5 *Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA*

Abstract

OBJECTIVE To generate monoclonal antibodies (MAbs) for developing a rapid malaria diagnostic urine-based assay (RUBDA), using *Plasmodium*-infected human urinary antigens.

METHODS *Plasmodium*-infected human urinary (PAgHU) and cultured parasite (CPfAg) antigens were used to generate mouse MAbs. The reactivity and accuracy of the MAbs produced were then evaluated using microplate ELISA, SDS-PAGE, Western blotting assay, microscopy and immunochromatographic tests.

RESULTS Ninety-six MAb clones were generated, of which 68.8% reacted to both PAgHU and CPfAg, 31.3% reacted to PAgHU only, and none reacted to CPfAg only. One promising MAb (UCP4W7) reacted in WBA, to both PAgHU and CPfAg, but not to *Plasmodium*-negative human urine and blood, *Schistosoma haematobium* and *S. mansoni* antigens nor measles and poliomyelitis vaccines.

CONCLUSION MAb UCP4W7 seems promising for diagnosing *Plasmodium* infection. Urine is a reliable biomarker source for developing non-invasive malaria diagnostic tests. SDS-PAGE and MAb-based WBA appear explorable in assays for detecting different levels of *Plasmodium* parasitaemia.

keywords malaria, *Plasmodium*, dipstick antigen monoclonal antibody

Introduction

Global eradication of malaria has not achieved expected success [1] partly because of the limitations in diagnostic accuracy [2, 3]. Also, tools presently available for malaria diagnosis are based on the invasive blood sampling procedures and sometimes involve the use of specialised techniques or equipment which are difficult to use outside the health facility [2, 4]. These limitations militate against home-based management of malaria, which has been identified as an important step towards eradication of malaria [5–8].

To overcome these limitations, this study explored the development of a rapid urine-based dipstick test (RUBDA) for diagnosis of malaria. For the RUBDA to function as an effective diagnostic tool, monoclonal antibodies (MAbs) are required for specific detection of malaria parasite antigens in urine.

MAbs for malaria diagnosis have been developed, primarily, from blood-based *Plasmodium* antigens [9–11]. Some of these MAbs have been used in simple RDT-based diagnostic tests for malaria, which include CareStart™ Malaria, Paracheck and Parasight *F*-test for detection of *P. falciparum* histidine-rich protein 2 [2, 3]. However, alternative human body fluids such as urine, which are obtained by less invasive procedures, are equally rich in malaria diagnostic antigens and could be used to produce more acceptable MAb-based tools for diagnosis of malaria. Yet, this field of R&D has not been fully explored.

In view of this, we aimed to generate MAbs against human urinary *Plasmodium* antigens suitable for developing a non-invasive, urine-based rapid diagnostic tool (RUBDA) for malaria.

Materials and methods

Setting

The study was conducted at Kpone-on-sea, a coastal fishing village in the Kpone-Katamanso District, Greater-Accra Region, Ghana, which has been GIS-mapped for malaria intervention studies. Kpone Health Centre (KHC) is responsible for healthcare services at Kpone-on-sea.

This was a cross-sectional study involving participants recruited from Kpone-on-sea. Negative control urine and blood samples were from uninfected individuals from the USA and were donated by courtesy of Prof. David Sullivan Jr. of the Johns Hopkins Bloomberg School of Public Health. Baltimore, MD, USA. Infected individuals were referred to the attending physician at KHC for treatment.

Ethical aspects

The objectives of the study were clearly explained to adult participants aged 18 years and older and to parents or guardians of younger participants. Written informed consent was obtained from adult participants and from the guardians of underage participants before the study began. The study protocol and ethical approval were obtained from the National Institutes of Health and Noguchi Memorial Institute for Medical Research (NMIMR) Scientific and Technical Committee and Institutional Review Board. The Committee for Human Research of the Johns Hopkins Bloomberg School of Public Health approved the analysis of urine samples in USA.

Participants

We recruited 1262 participants aged 5 months to 90 years into the study, of whom 38.59% were males. About 51.03% comprised children aged 6–15 years, of whom 50.31% were females; 17.91% were children below age 6, of whom 47.80% were female.

The clinical history of each study participant together with age, sex and body temperature was taken before sample collection. Subjects were eligible for the study if they were diagnosed with *Plasmodium* species and had not taken any antimalarial chemotherapy prior to sample collection. Axillary temperature was taken using a digital thermometer before sample collection.

Field procedures

For the preliminary analysis, 20–150 ml of fresh clean catch urine was collected from each study participant between 08:00 and 14:00 h GMT into a 150-ml urine container before administration of antimalarial chemotherapy. The urine samples were transported to the main laboratories at University of Ghana, Legon, for processing.

Thick and thin blood smear preparations and examinations for parasite density and speciation were done as previously described [12]. Examined blood smear slides were transported to the NMIMR Laboratory for quality control checks.

After blood smear preparation, a separate fraction of blood sample from each participant was also tested using the quantitative buffy coat (QBC) technique [13–15].

Blood samples from participants were also screened for malaria-related antigens as described for urine samples below with minor changes. Briefly, a drop of blood was dispensed into the sample well of each test cassette using the micropipette provided, according to the manufacturers' specifications. Haemolysing washing buffer was then added to remove the haemoglobin and allow visualisation of coloured positive and control test lines.

Laboratory procedures

Part of each urine sample arriving at the NMIMR was aliquoted into a 14-ml Falcon centrifuge tube and stored frozen at −20 °C until used. Fractions of the urine samples from malaria microscopy or QBC-positive individuals were tested by rapid diagnostic tests strips as described below, for the presence of *Plasmodium* antigens. *Plasmodium* antigens (CPfAg) and (PAgHU), extracted from *in vitro* cultured 3D7 *P. falciparum* parasites and infected human urine, respectively, were tested for *Plasmodium* antigens according to the method described by the manufacturers (AccessBio Inc., Somerset, NJ, USA) with modification. Briefly, 20 µl aliquots of *Plasmodium* antigen extracts or unprocessed urine samples from malaria microscopy-negative and positive individuals was dispensed into the sample wells of rapid diagnostic test kit cassettes {CareStart™ Malaria HRP-2 (Pf) or HRP-2/pLDH (Pf/pan) Combo RDT} (AccessBio Inc.) for testing. The wells were then allowed to develop for 15 min at room temperature (22–25 °C). Positive dipstick test results showed a horizontal reddish brown line in addition to a control line on each test strip in a distinct detection zone. Negative results showed only the positive control line.

Urine samples from >400 infected subjects were pooled for antigen extraction. Samples were eligible for pooling if they were positive by HRP-2 or LDH-based RDT and their corresponding blood samples were also positive by blood smear microscopy or QBC test. Isolation of *Plasmodium* proteins from urine samples was performed by precipitation with 33–50% saturated ammonium sulphate

solution and centrifugation as described in the Farr Technique protocol [16, 17]. Urinary antigens extracted were confirmed for the presence of *Plasmodium*-related proteins using RDT and microplate ELISA.

The concentration of proteins in the urinary or cultured *Plasmodium* antigen extract was estimated using the Bradford protein assay technique [18].

Laboratory strain *P. falciparum* parasites were cultured [19, 20]. Infected RBCs were harvested from culture for antigen extraction when parasite density was 8–10% [21]. Infected RBCs containing schizont-stage parasites were separated from uninfected RBCs [22] and then lysed [21].

BALB/c mice were immunised as described by [23] with slight modification. Briefly, CPfAg and PAgHU were used as immunogens. They were administered at the rate of 15 μg/100 μl or 30 μg/200 μl of immunogen per mouse per inoculation. Mice were immunised via the peritoneal route [24–26].

In vitro propagation of myeloma cells, cell fusion and selection of MAb secreting hybridoma cells were carried out as described elsewhere [23].

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) on proteins in different fractions of urine and cultured *Plasmodium* crude antigen extract was conducted using the SDS tris-glycine discontinuous buffer system described by [27]. After SDS-PAGE, the gel was either stained with Coomassie blue dye for estimation of molecular weight (MW) of proteins or examined by Western blotting analysis. The MWs of the various proteins were estimated [28].

After SDS-PAGE, Western blotting assay was carried out to determine the reactivity of separated proteins to MAbs produced using the chemiluminescent detection method described by Mathews et al. [29].

Results

Prevalence of *Plasmodium* infection

Of the 1262 participants tested by non PCR-confirmed blood smear microscopy (Table 2), 46.51% were positive (geometric mean parasitaemia = 55466.10598).

Immune response in BALB/c mice

Figure 1a and b summarises the response of mice to immunisation with PAgHU and CPfAg as determined by microplate ELISA. As shown, generally, all mice exhibited a higher immune response to PAgHU than to CPfAg. Mice numbered 6, 11 and 13 showed good immune response to both PAgHU (ODs = 2.51, 1.25 and 2.45) and CPfAg (ODs = 0.79, 1.6 and 0.55), respectively. Consequently, these three mice were selected as the most suitable for cell fusion to generate hybridoma cells for monoclonal antibody production.

Reactivity of MAbs generated to urinary and cultured parasite antigens

In all, 96 clones of mouse anti-*Plasmodium* MAbs were generated after cell fusion. As summarised in Table 1, whereas all 96 (100%) MAb clones were reactive to PAgHU, only 68 (70.8%) reacted with CPfAg. In terms of strength of reactivity to PAgHU, 10 of 96 (10.4%) clones were hyperpositive (3+), 20 (20.8%) were strongly positive (2+), and 48 (50.0%) were moderately positive (+). In contrast, only 1 (1.0%), 3 (3.1%) and 16 (16.7%) clones, respectively, showed the respective reactivity to CPfAg (Table 1). Regarding weakly positive and negative reactivity, however, fewer MAbs were reactive to PAgHU (18.8% and 0%) than to CPfAg (50.0% and 29.2%), respectively.

UCP4W7 monoclonal antibody

UCP4W7 represents Urine Culture Plate #4 Well #7. UCP4W7 MAb has an IgG1 isotype. It was generated by immunising BALB/c mice with *Plasmodium*-infected human urinary and cultured parasite antigens. UCP4W7 could bind to both urinary and cultured *Plasmodium* antigens in ELISA and WBA. Even though it has not yet been tested against other species of the parasite, PAgHU that this MAb is specific to contains *P. falciparum*-specific HRP-2 and *Plasmodium* pan-specific LDH antigens.

Isolation of proteins in different antigen preparations

Figure 2a and b shows the results of protein separation in different antigen preparations by SDS-PAGE. As shown, PAgHU (Figure 2a, lane 2 or 2b, lane 4) and malaria-negative blood sample (MNBS) (Figure 2a, lane 3, or 2b, lane 5) had more (>10) clearly visible protein bands each than the rest of the various antigens separated. These proteins included those of MWs, 27.2, 32, 52 and 63.2 kDa. Also, yellow fever vaccine (Figure 2b, lane 9) apparently had >10 proteins, most of which merged to form a smear.

The malaria-negative urine sample from Ghana (Figure 2a, lane 6 or 2b, lane 8) had the least number (2) of clearly visible proteins with estimated molecular weights between 63.2 and 75.4 kDa.

Finally, the results showed that proteins B and E were relatively the most prominent bands in the gel.

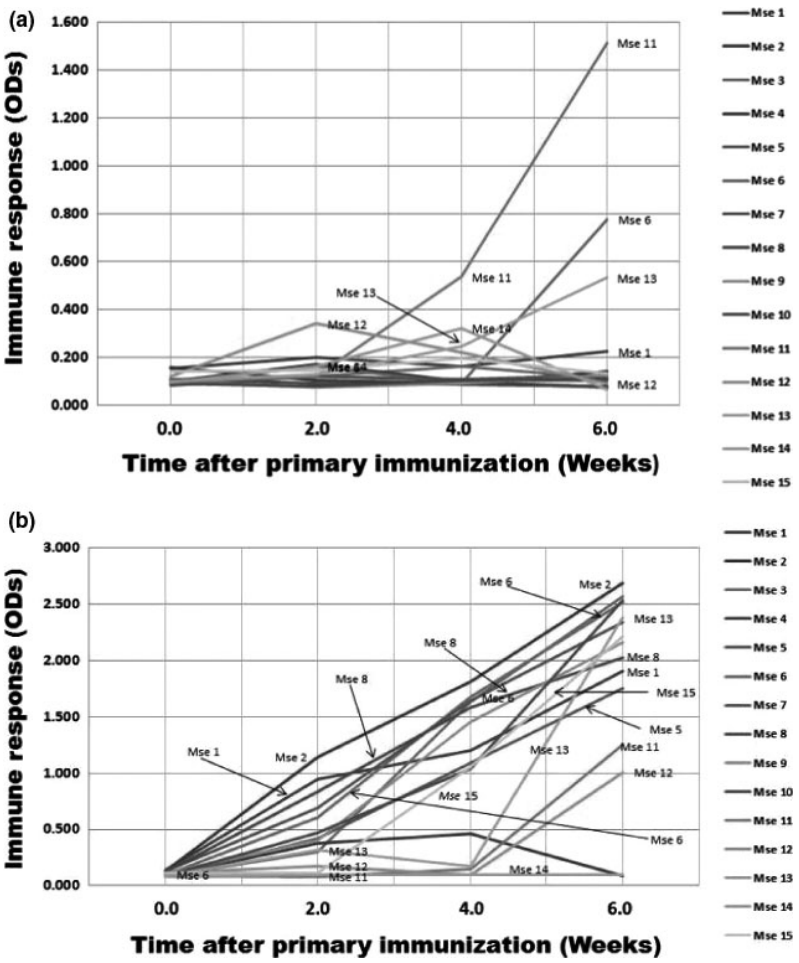

Figure 1 (a) Immune response of BALB/c mice to urinary *Plasmodium* antigens. (b) Immune response of BABLB/c mice to cultured *Plasmodium* antigens. Mse, mouse.

Table 1 Reactivity of anti-*Plasmodium* monoclonal antibodies by microplate ELISA

Reactivity strength		No. of monoclonal antibody clones reactive to	
Name	Grade	PAgHU No (%)	CPfAg No (%)
Hyperpositive	3+	10 (10.4)	1 (1.0)
Strong positive	2+	20 (20.8)	3 (3.1)
Moderate positive	+	48 (50.0)	16 (16.7)
Weak positive	+/−	18 (18.8)	48 (50.0)
Negative	−	0 (0.0%)	28 (29.2)
Total		96 (100.0)	96 (100.0)

Reactivity of UCP4W7 MAb

Ten (10) of the 96 MAbs generated showed hyperpositive (3+) reactivity in microplate ELISA, with absorbance OD ranging from 2.75 to 3.10 at 414 nm, and were selected for further analysis by a preliminary Western blotting assay (WBA). However, only one (1) of the 10 MAbs examined showed strong reactivity, five showed weak reactivity and the remaining four showed no reactivity by WBA. The one (1) MAb (UCP4W7) that showed strong reactivity, both in microplate ELISA and in the preliminary WBA was, therefore, selected for further analysis for cross-reactivity to other parasite antigens using WBA.

Figure 3a and b shows the results of the reactivity of UCP4W7 MAb with different antigen preparations as determined by Western blotting assay. UCP4W7 MAb was reactive to PAgHU, CPfAg and DUMIs, but not to *Plasmodium* antigen-negative urine or blood samples. Of the proteins detected by UCP4W7 MAb in PAgHU, four were very sharp and comprise proteins C, D, E and F with estimated MWs, 27.2, 32, 63.2 and 75. 4 kDa,

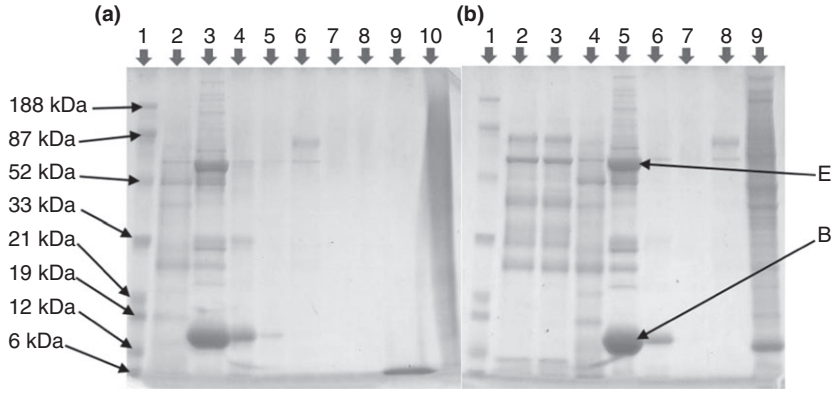

Figure 2 (a and b) Analysis of proteins in different antigen preparations by SDS-PAGE. Note (a): 1 = Molecular weight marker, 2 = Human urinary *Plasmodium* antigen extract (PAgHU), 3 = Malaria-negative blood sample (MNBS), 4 = Cultured *P. falciparum* crude antigen (CPfAg), 5 = *Plasmodium*-negative urine from USA (JHU-Ve), 6= *Plasmodium*-negative urine from Ghana (GH-Ve), 7 = *Schistosoma mansoni* soluble egg antigen (*Sm*. SEA), 8 = *Schistosoma haematobium* adult worm antigen (*Sh*. AWA), 9 = Polio vaccine, 10 = Measles vaccine. Note (b): X = Molecular weight marker on Coomassie blue gel, 1 = Molecular weight marker, 2 = *3+ *Plasmodium*-positive urine, 3 = $^{\Phi}$4+ *Plasmodium*-positive urine, 4 = Human urinary *Plasmodium* antigen extract (PAgHU), 5 = Malaria-negative blood sample (MNBS), 6 = Cultured *P. falciparum* crude antigen (CPfAg), 7 = Malaria-negative urine from USA (JHU−ve), 8 = Malaria-negative urine from Ghana (GH−ve), 9 = Yellow fever vaccine, *3+ = 50–100 000 parasites/μl of blood, $^{\Phi}$4+ = >100 000 parasites/μl of blood, Estimated molecular weights of proteins in kDa, A = <6; B = 13.5; C = 27.2; D = 32; E = 63.2; F = 75.4.

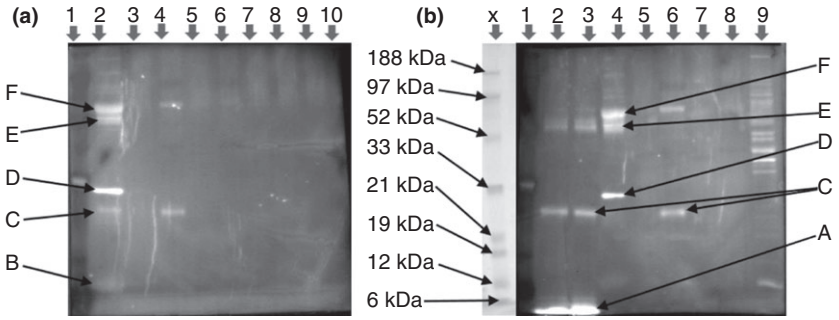

Figure 3 (a and b) Analysis of proteins in different antigen preparations by UCP4W7 MAb-probed Western blotting assay. Definitions of (a) and (b), refer Notes (a) and (b) in Figure 2, respectively.

respectively. Whereas proteins C, E and F were also detected in the cultured *Plasmodium* parasite, antigen (CPfAg) D was not.

The UCP4W7 MAb also reacted with more than three similar proteins in unprocessed urine samples from the 3+ and 4+ malaria-positive individuals (DUMIs) (Figure 3b). The most prominent of these proteins were A (MW <6 kDa), C and E. Whereas A was present only in the DUMIs, C and E were also present in PAgHU and CPfAg as described above.

Furthermore, there were several bands within the yellow fever vaccine protein profile (Figure 3b, lane 9), which reacted non-specifically with the UCP4W7 MAb. Lastly, there were no proteins in the malaria-negative blood or urine samples that showed observable reactivity to the UCP4W7 MAb.

Discussion

The primary aim of this study was to generate monoclonal antibodies (MAbs) against urine-based *Plasmodium* antigens and evaluate their reactivity for development of a rapid urine-based dipstick assay (RUBDA) for diagnosis of malaria. Current confirmatory diagnostic tests for malaria are blood-based [30–35], less sensitive and specific. Also, they cannot be performed in many field environments because of lack of technical expertise, electricity, temperature control, storage and laboratory equipment.

MAb-based dipstick tests requiring minimal or no technical expertise have been produced for prompt and easier diagnosis of malaria [30, 32–34], but all commercially available MAb-based dipstick tests are blood-based [30]

and cannot be used for home-based management of malaria, a major strategy for reduction of malaria morbidity and mortality [5–8]. The generation of urinary anti-*Plasmodium* MAbs suitable for the development of simple non-invasive tests for malaria, therefore, is of utmost importance to improve malaria diagnostic coverage necessary for reduction of malaria morbidity and mortality.

Also, the characterisation of MAbs generated for reactivity to *Plasmodium* antigens in human urine and cross-reactivity to antigens of different parasites is essential to ensure their specificity against urinary *Plasmodium* antigens and development of an accurate RUBDA for control of the disease. To achieve these goals, we therefore produced MAbs against *Plasmodium* antigens (PAgHU) from infected human urine and cultured 3D7 parasites (CPfAg) and evaluated their reactivity and accuracy using microplate ELISA, SDS-PAGE, Western blotting assay, blood smear microscopy, quantitative buffy coat and immunochromatographic tests.

In all, 96 clones of MAbs were produced after cell fusion. The superior reactivity (100%) of the MAbs to PAgHU than to CPfAg (70.8%) corroborates the report [36] that urinary antigen extracts are more immunogenic than soluble antigens from whole parasites. Also, the detection of both PAgHU and CPfAg by 68 (70.8%) of the MAbs suggests that they have a wider range of diagnostic applicability and could, therefore, be the most promising for development of the urine-based diagnostic test for malaria.

High relative sensitivity was observed for urinary PfHRP-2 RDT (96.6%), urinary LDH RDT (98.0%) and UCP4W7 MAb ELISA (96.8%) compared to microscopy as gold standard test at detecting *Plasmodium* infection in the participants (Table 2); however, the specificities, 64.6, 68.4 and 75.7%, respectively, were significantly lower ($P < 0.01$). The rather low relative specificities of these tests could be due to either low sensitivity of the microscopic technique or high false positivity rates of these serological tests. The first explanation is more likely to be correct because, although blood smear microscopy is presently the established method for confirmation of malaria [37], several studies [38, 39] have shown that it is not sensitive enough, and often leads to under estimation of malaria prevalence.

Indeed, in a more recent study, using the improved PCR technique that enables detection of submicroscopic *Plasmodium* parasite infection [40], it was reported that approximately 21% of 101 blood samples that tested negative by blood smear microscopy tested positive by the improved PCR technique. However, these 101 blood samples were collected from our Kpone-on-sea study community, prepared and examined microscopically by the same qualified technicians who assisted in the processing and analysis of the 1262 blood samples whose results are being discussed in this current paper. Thus it was not surprising that a higher ($P < 0.01$) prevalence of *Plasmodium* infection was obtained by the other tests among the participants than by microscopy.

Table 2 Sensitivity and specificity of tests for different biomarkers of malaria compared with blood smear microscopy as the gold standard

Test	Number tested	Number positive	Number negative	Relative sensitivity (%)	Relative specificity (%)
Blood smear microscopy*	1262	587	675	100	100
Blood-based HRP2 ICT†	1262	704 (250)[a]	558 (100)[b]	77.3	67.9
QBC test‡	1262	657 (193)[a]	605 (90)[b]	79.1	76.3
Urinary PfHRP2 ICT§	1262	697 (130)[a]	565 (129)[b]	96.6	64.6
Urinary LDH ICT¶	1262	680 (105)[a]	582 (120)[b]	98.0	68.4
UCP4W7 MAb ELISA**	1262	721 (153)[a]	541 (30)[b]	96.8	75.7

*Gold standard test.
†Diagnosis of *Plasmodium infection in blood* by CareStart™ malaria RDT.
‡Diagnosis of *Plasmodium* infection by quantitative buffy coat test.
§Detection of *Plasmodium* HRP2 antigens in urine by CareStart™ malaria RDT.
¶Diagnosis of *Plasmodium* pan-specific LDH antigens in urine by Combo CareStart™ malaria RDT.
**Detection of *Plasmodium* antigens in urine UCP4W7 MAb-based microplate ELISA.
[a]False positives (number positive by other tests that tested negative by microscopy).
[b]False negatives (number negative by other tests that tested positive) by microscopy).
Relative sensitivity and specificity of each test were calculated using the formulae: Relative sensitivity of test = {(V−a)/U} × 100%; Relative specificity = {(Y−b)/ W} × 100%, where: U = Number positive by microscopy; V = Number positive by other tests; W = Number negative by microscopy; Y = Number negative by other tests.

Although UCP4W7 MAb ELISA had a significantly lower relative specificity than microscopy, the fact that its combined reactivity (relative sensitivity and specificity = (96.8 + 75.7)% = 172.5%) is >170% suggests that it is more accurate for diagnosis of *Plasmodium* parasite infections than the other tests [41].

From these findings, the rather interesting performance of some of the pre-existing commercial blood-based RDT at detecting *Plasmodium* infection in the urine of infected individuals suggests that perhaps it is time some of them were re-evaluated and, if proved to be promising, were endorsed for routine urine-based diagnosis of malaria.

The positive reactivity of the UCP4W7 MAb in Western blotting to *Plasmodium* antigens in urine (PAgHU), cultured parasites (CPfAg) and the direct urine from infected individuals (DUMIs), but not with *Plasmodium*-negative blood or urine samples from Ghana and USA, suggests that UCP4W7 MAb could be used to distinguish between malaria-positive and malaria-negative individuals.

Also, the detection of proteins B (13.2 kDa) and D (32 kDa) by UCP4W7 in PAgHU, but not in either CPfAg or DUMIs, suggests that probably, the strains of the *Plasmodium* parasites that produced antigens B and D in PAgHU, do not have these proteins in common with those used in producing CPfAg and DUMIs [42–45]. Indeed, this uniqueness in antigenic properties of PAgHU might have occurred because urine samples from over 400 infected participants had to be pooled together in order to prepare PAgHU.

The absence of antigens B and D from the negative samples suggests that probably, these proteins originated from the *Plasmodium* parasite and were not detected in the negative samples because the donors did not have the infection. This observation suggests that UCP4W7 is capable of accurately distinguishing between negative and positive infections.

Various authors [46, 47] reported that the strength of reactivity (prominence) of proteins in western blotting is directly proportional to their amount or concentration in the sample preparation. In view of this, the detection of the 75.4-kDa protein, labelled F, with reducing reactivity strength from PAgHU to CPfAg to DUMI-2 (>100 000 parasites/μl of blood) and then to DUMI-1 (50 000–100 000 parasites/μl of blood), but not in all the negative samples, suggests that perhaps, F was a *Plasmodium* parasite protein whose concentration in the urine was probably associated with parasite density. The reduction in reactivity to F with parasite density, therefore, indicates that protein F could be a useful biomarker for semiquantitative estimation of parasite density in infected individuals. Further studies are required to validate this observation.

Proteins E (63.2 kDa) and F (75.4 kDa) had more intense and broader bands than all other proteins in PAgHU suggesting that they had higher concentrations than the other proteins in PAgHU, as reported by Goins [47]. In their quantitative analysis of complex protein mixtures, Gygi et al. [48] found that measured differences in protein expression in yeast correlated with their metabolic functions under glucose-repressed conditions. As the levels of expressed proteins are directly related to their concentrations or relative abundance in a biological sample, proteins E and F, which showed more intense and broader bands than the others, may play more important biological roles within the *Plasmodium* parasite by implication [49].

Of the non-malarial proteins examined for cross-reactivity, yellow fever vaccine reacted nonspecifically to UCP4W7 MAb. It is, however, important to note that owing to logistics constraints, we had only one vial of this vaccine to use in this study and cannot draw any reliable conclusion from this observation. Further studies are underway with additional antigens to investigate extensively the cross-reactivity of UCP4W7 MAb to nonplasmodial antigens including yellow fever. Viral antigens were included in the cross-reactivity studies because they are also excreted in the urine of infected or vaccinated individuals [50, 51] and could limit the accuracy of urine-based malaria diagnosis by UCP4W7 MAb.

The utilisation of blood-based commercial malaria RDT kits for ascertaining the presence of *Plasmodium* antigens in infected human urine samples before extraction of antigens for immunisations, however, is a limitation of this study.

Conclusions

This study has shown that UCP4W7 MAb could be used to develop a non-invasive urine-based test for malaria, and a semiquantitative urine-based test for assessment of malaria parasite density in infected individuals.

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Corresponding Author Uri S. Markakpo, Department of Biological, Environmental & Occupational Health Sciences, School of Public Health, University of Ghana, PO Box LG13, Legon, Accra, Ghana. Tel.: +233 28 9109002; Fax: +233 302 517501; E-mail: umarkakpo@ug.edu.gh