1-1-2019

Average Time To Negativity Of New Alere Ultra-Sensitive Rapid Diagnostic Test After Treatment For Malaria.

Azan Zahir Virji
azan.z.virji@gmail.com

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A thesis presented to:

The Department of Epidemiology of Microbial Diseases

Yale School of Public Health

In partial fulfillment of the requirements for the degree of:

Master of Public Health (MPH)

By:

Azan Zahir Virji

May 2019

Advisor/Committee Chair: Sunil Parikh, MD, MPH

Committee Members: Amy Bei, PhD

Abstract

Malaria remains one of the most challenging infections globally. Malaria RDTs have had the largest impact on malaria detection in recent years with almost 245 million RDTs delivered globally. (1) They work by detecting either the histidine-rich protein (HRP2) or the *Plasmodium* lactate-dehydrogenase (pLDH). (2) Following a malaria infection, the HRP2 and pLDH antigens often circulate in the blood stream for several weeks. (3) As new RDTs are constantly being developed, each with a better sensitivity, specificity and lower limit of detection than its predecessor, the need to understand the persistence of their positivity, following a malaria infection, remains. The latest RDT on the market is produced by Alere™ and is called the ultra-sensitive RDT (uRDT). To test how long this new RDT remains positive following a malaria diagnosis and treatment, we prospectively followed children for 42 days. Children were either HIV positive or negative and either placed on a 3-day or 5-day artemether-lumefantrine regimen. Microscopy and the uRDT were performed on days 0, 7, 14, 21, 28, 35, and 42. The mean length of uRDT positivity was 9.2 days with a standard deviation of 11.3 days. Approximately 50% of all uRDTs remained positive for 11 days post treatment of malaria. Neither HIV status nor AL duration impacted the length of uRDT positivity. Only parasite density was directly correlated with uRDT positivity with an increase in parasite density of 1 parasite/μl resulting in 0.6 additional days of uRDT positivity. Microscopy (considered the gold standard in our study) and uRDT outcomes matched 67.5% of the time (320/474). The true positive rate (sensitivity) was calculated to be 91.3% and the true negative rate (specificity) was 55.3%. The uRDT was also able to predict treatment failure in children 7 days prior ($p = 0.015$). Due to its positive persistence that can lead to false positive, we recommend that the use of Alere's uRDT be limited to low transmission and elimination settings only.

Acknowledgements

Throughout the writing of this thesis, I have received extensive support and assistance. For that, I would like to thank my thesis supervisor, Dr. Sunil Parikh, for his guidance and mentorship. I would also like to thank Dr. Amy Bei for her feedback and comments that have helped shape this thesis.

I would also like to acknowledge colleagues in Tororo, Uganda, that have spent countless hours recruiting participants and collecting data. Thank you to Dr. Mbezwa, Dr. Richard and Dr. Francis and the entire team at the Infectious Disease Research Collaboration (IDRC).

I am grateful to the National Institutes of Health (NIH) for funding this study.

Finally, to God, my parents and all my other teachers/professors, you are the reason I am here today.

"Struggle is the meaning of life; Defeat or victory is in the hands of God; but struggle itself is a man's duty and should be his joy"

Sir Sultan Muhammed Shah, Aga Khan III

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Average time to negativity of new Alere™ ultra-sensitive rapid diagnostic test after treatment for malaria.

Azan Zahir Virji [1] Sunil Parikh MD, MPH [1] Norah Mbezwa MBChB [2]

1: Yale School of Public Health, Department of Epidemiology of Microbial Diseases, New Haven, Connecticut, 06510

2: Makerere University, Kampala, Uganda

Introduction

Malaria remains one of the most challenging infections globally. In 2017, it is estimated that there were approximately 219 million cases with 435,000 deaths. The global community has made significant strides in malaria control over the past 2 decades, with most of the strides resulting from improved case detection, treatment, and prevention with bed nets. However, recent years have seen these gains stagnate or reversed in many areas. The 10 highest burden countries reported increases in malaria cases with over a half a million more cases in Nigeria, Madagascar and the Democratic Republic of Congo. (1) Efforts to thwart the spread of the disease have relied predominantly on improved diagnosis and treatment of patients (4). Getting back on track to achieve the 2025 milestones, will require a concerted effort to expand our malaria detection capabilities and ensure no case goes untreated.

Currently, three main methods of malaria parasite detection are employed: 1) light microscopy of stained blood smears for physical observation of parasites; 3) nucleic acid amplification methods of parasite DNA; 3) immunochromatographic tests, such as rapid diagnostic tests (RDTs) or rapid antigen tests (RATs) for detection of parasite proteins. Each method of diagnosis presents its own advantages and challenges.

Light microscopy

Since its development in 1904, light microcopy following Giemsa or Wright staining of thick or thin blood smears, has remained the most suitable and convenient diagnostic method for malaria. (5) In 2017, 25% of all malaria diagnosis was done using light microscopy. (1)

Following the initial purchase of a microscope (~$300) each malaria smear is relatively inexpensive (~ $0.12-$0.40). (5, 6). While most microscopes still need electricity, the invention of high-quality light emitting diodes (LED) illumination and solar battery chargers has made microscopy more feasible in remote areas. (5) Moreover, microscopy is also able to differentiate between malaria species and quantify parasitemia. (5) **(Supp. Fig. 1)**

However, light microscopy is still limited by its need for well-trained microscopists and its relatively poor diagnostic sensitivity. (4, 5) Skilled personnel are required to properly prepare smear slides. Low-quality blood film generation can lead to artifacts, such as bacteria, fungi, stain precipitation and cell debris, being commonly mistaken for malaria parasites. (5) Additionally, failure to distinguish between *P. falciparum* from *P. vivax,* the two most common species, is frequent, underreported and due to poor training. (5) Currently, the limit of detection

(LOD) for microscopy is estimated to be between 4-20 parasites/μl in lab settings and 50-100 parasites/μl in field settings, a threshold that is likely higher in areas with inadequate equipment and poorly-trained microscopists. (4, 5) As efforts to improve malaria microscopy ramp up, it continues to remain the gold-standard for confirmation of an active infection, especially in low-resource settings. (4, 5)

Nucleic acid detection

Molecular methods to diagnose malaria include end-point polymerase chain reaction (PCR), real-time quantitative polymerase chain reaction (qPCR) or the more recently optimized method, Loop-mediated isothermal amplification (LAMP) (7). PCR for malaria detection relies predominantly on the detection of the 18S RNA gene because it is specific, conserved in all *Plasmodium* species, and has ~5-7 copies per genome making it a highly sensitive test. (8) DNA targets for PCR have also been developed but are considered inferior because the circulation of DNA post-infection can lead to false positives. (9) On average, the threshold for detection for all PCR based methods is 0.1-5 parasites/μl. (8-10) With the requirement of only 5μl of blood, PCR is by far the most accurate method of malaria parasite detection.

Despite its high diagnostic accuracy, one the biggest challenges with PCR is its cost and inability to be performed at point of care (POC). (10) However, LAMP may offer a practical alternative. Designed by the Foundation for Innovative New Diagnostics, Switzerland, and Eiken Chemical Co., Ltd., Japan, the vacuum-dried reagents that are stable at ambient temperature allow LAMP to be used in resource-constrained settings. (10) Like PCR, it also amplifies nucleic acids (LOD 0.2-2 parasites/μl) but uses simpler equipment, such as a heat-blocks instead of a thermocycler

and is less time intensive than regular PCR. (10-12) Furthermore, LAMP reagents are also cheaper than conventional PCR reagents ($0.40-$0.70 and $1.50-$4 respectively). (9) Although no quantitative results can be derived from LAMP and results must be read with the naked eye using a UV light, LAMP is a promising method of POC diagnosis of malaria. (9, 11, 12) **(Supp. Fig 1)**

A study to test the performance of LAMP kits in a remote clinic in Uganda highlighted two key insights: 1) that a sensitivity similar to that of a nested PCR in a United Kingdom reference laboratory (with a qualitative reading by gel) could be achieved using LAMP in the field setting and 2) technicians without previous molecular training could reliably perform LAMP following a short training period (10, 12). However, even though the LAMP reactions proceed at a much lower temperature of ~60°C compared to PCR, electricity is still needed to run the test creating a barrier to point of care detection (13).

Rapid diagnostic tests

Malaria RDTs have had the largest impact on malaria detection in recent years. In 1990, the first malaria RDTs were introduced to overcome the practical challenges of microscopy with over 60 manufacturers supplying the tests by 2005 (14). In 2017, almost 245 million RDTs were delivered globally. (1) A large majority of these RDTs (66%) were capable of only detecting *P. falciparum* and were delivered to the WHO African Region (1). Today, the WHO recommends that all clinically suspected cases of malaria be confirmed either through the use of microscopy and/or RDT, both of which should be available at testing sites (15).

Malaria RDTs work by detecting either the histidine-rich protein (HRP2), only found in *Plasmodium falciparum*, or the *Plasmodium* lactate-dehydrogenase (pLDH) found in all *Plasmodium sp.* (2). The HRP2 is a water-soluble protein produced by trophozoites and young (not mature) gametocytes whereas pLDH is produced by both asexual and sexual stages of malaria parasites. (16) Following a malaria infection, the HRP2 and pLDH antigens often circulate in the blood stream for several weeks. (3) A study modelling the length of RDT positivity found that 50% of treated individuals would still present a positive HRP2 RDT after 15 days (5-32 95% CI) and a positive pLDH RDT after 2 days (1-7 95% CI). (7, 17, 18). This often misconstrues whether or not a new infection is present and can lead to a false positive malaria diagnosis, particularly in settings where transmission intensity is high. Similarly, cross reaction with other infections such as schistosomiasis and trypanosomiasis can also occur in areas where these diseases are prevalent. (19, 20).

Another limitation of RDTs includes the number of false negatives due to deletions of the HRP2 gene or parasitemia levels below the LOD (7, 21-23). In 2010, malaria parasites with the *pfhrp2* gene deletion were confirmed in the Amazon Basin in Peru. (23) Since then, multiple reports have confirmed false negatives and *pfhrp2* PCR-negative parasites in 9 countries across the African continent. (24, 25) In Mozambique, a molecular surveillance study found that out of the 69 individuals who were RDT negative but microscopy positive, only one (1.45%) had a *pfhrp2* deletion. (24) False negatives can also be caused by poor transport and storage with sustained exposure to high temperatures (23) **(Supp. Fig 1)**

In spite of these limitations, RDTs are an inexpensive ($0.45-$1.50) and highly effective way of detecting malaria in a relatively short period of time (5-20 mins), with limited-training and in low prevalence areas (5, 7, 9, 18). A meta-analysis of RDTs estimate an average sensitivity and specificity of 95.0% (95% CI: 93.5-96.2%) and 95.2% (95% CI: 93.4-99.4%) respectively for *Pf*HRP2 (7). RDTs detecting *Pf*-pLDH report a marginally lower sensitivity of 93.2% (95% CI: 88.0–96.2%) but higher specificity of 98.5% (95% CI: 96.7–99.4%) (7). With an overall LOD of approximately 5-200 parasites/µl and no need for laboratory infrastructure, RDTs are considered a quick yet accurate method of detecting malaria even in remote settings. (3, 26)

Ultra-sensitive RDT

New RDTs are constantly being developed, each with a better sensitivity, specificity and LOD than its predecessor. The latest RDT on the market is produced by Alere™ and is called the ultra-sensitive RDT (uRDT).

Using the same volume of blood (5-15µl) and the same immunochromatographic cassette as other RDTs, the uRDT is supposedly 10 times more sensitive than current malaria RDTs with a lower LOD of 0.1-100 parasites/µl (2, 27, 28). The uRDT differs from the Standard Diagnostics, Inc. BIOLINE Malaria Ag P.f RDT (SD-RDT) since it uses carboxyl-modified and biotinylated latex fragment antibodies (FAbs) and polystreptavidin on the test line (26). However, both the uRDT and the SD-RDT use lateral flow and 5 µL of whole blood specimen (WBS) (26, 28, 29).

When compared to a widely used SD- RDT, the uRDT has shown an increased sensitivity in detecting asymptomatic parasitemia (29, 30). Using qPCR as the gold standard, the uRDT had a

sensitivity of 84% while the SD-RDT had a sensitivity of 44% in the high-transmission setting of Uganda. On the other hand, the uRDT had a sensitivity of 62% and the SD-RDT had a sensitivity of 0% in the low-transmission setting of Myanmar. (30)

Alere's uRDT will undoubtedly serve as a useful tool in the elimination of malaria in low transmission areas. However, it is likely that as the advantages of the uRDT become more apparent, it will slowly replace other RDTs, even in high transmission settings. In anticipation of this, we set out to understand how long the uRDT remains positive for post anti-malarial treatment and compare the uRDT to microscopy in the malaria endemic setting of Uganda. Since the HRP2 antigen circulates in the blood following a malaria infection, it is likely that the uRDT will remain positive for an extended period of time post-treatment and result in a large number of false positives. To test this hypothesis, a longitudinal cohort of children in Tororo, Uganda who were either HIV positive or HIV negative, were placed on a 3-day or a 5-day Artemether-Lumefantrine (AL) combination therapy and followed prospectively for 42 days. A uRDT and microscopy test were performed at regular 7-day intervals between day 0-42. Time to negativity for the uRDT was analyzed and whether HIV status or duration of AL therapy had any impact on this time. Finally, we assessed if the uRDT was able to pick up treatment failures (TFs) in children before they occurred.

Materials and Methods

Study site

Uganda is among the 15 countries most affected with malaria in the world with the predominant malaria species being *Plasmodium falciparum* (31). Of the 16 countries globally that are responsible for 80% of the global malaria burden, Uganda ranks fourth (1). The country has an annual rainfall of 1,000 – 1,500 mm typically between the two rainy seasons (March – May and August – October) (32). Between 2016 and 2017, Uganda saw an increase in 25% of reported malaria cases (8,600,724 cases with 14,390 malaria-related deaths in 2017) (1). Seven percent of all deaths in children under 5 and 11% of all deaths in post-neonates were due to malaria in 2016. (33) The average malaria parasite prevalence in Uganda is 45% and follows a perennial transmission pattern (34, 35). Hospital records predict that 30-50% of all outpatient visits and 9-14% of all inpatient deaths are due to malaria (34).

Tororo is located in a savannah grassland at an elevation of 1,185 m above sea level in Eastern Uganda (32). Maize and rice form the staple of the diet and are grown on low-lying wetlands, a perfect breeding ground for mosquitoes (31, 32). The major mosquito species that dominate the region include *Anopheles gambiae s.s.* and *Anopheles funestus* with some *Anopheles arabiensis* (32). In 2012, Tororo had the highest burden of malaria in the country of Uganda with a *Plasmodium* prevalence of 48% (31). The annual entomological inoculation rate (aEIR) for *Plasmodium falciparum* transmitted by *Anopheles gambiae* in 2012 was 125 (95% CI: 72.2 – 183.0) (32). The most recent estimate in 2015 puts the malaria parasite prevalence in Tororo at 18%. (36)

Busia sits under Tororo at an elevation of 1,198m with half of the district crossing the border into Kenya (37). Across Uganda, mortality due to severe malaria is highest in Busia (38). With a meager population of 55,958, Busia saw almost 54,652 *P. falciparum* cases of malaria in 2015 out of 7,111,817 cases across the country of Uganda. (39, 40)

The study was conducted in the eastern region of Uganda in the Tororo and Busia districts at the Tororo District Hospital (TDH) or Masafu General Hospital (MGH) between February and December 2018. **(Fig. 1)**

Study methodology

Our study was done in the context of an NIH-funded trial, named Extended Duration Artmether-Lumefantrine (AL) Treatment for Malaria in Children (EXALT), a collaboration between Yale, University of California San Francisco (UCSF) and the Infectious Disease Research Collaboration (IDRC). This parent study is a prospective cohort study looking at the pharmacokinetic and pharmacodynamic changes following an extended duration of AL in HIV-infected children on EFV and HIV-uninfected children.

Enrollment consisted of 160 children, aged 3-10 years who were HIV positive (HIV+) and on EFV-based ART, in addition to 220 children aged 6 months-10 years who were HIV negative (HIV-). Inclusion criteria included: 1) residency within 60 km of the study clinics; 2) Weight ≥ 6 kg; 3) A microscopically and clinically confirmed case of uncomplicated *P. falciparum* malaria; 4) Provision of informed consent and willingness to return to the clinic for evaluations.

Within each group, based on HIV status, children were randomized to either a 3-day AL therapy or a 5-day AL therapy. They were then followed for 42 days following their initial malaria diagnosis to assess the incidence of a recurrent malaria episode. The two main primary outcomes were the measurement of the plasma concentration of all drug analytes (independent variables) and the recrudesce or new infection of malaria (dependent variable).

For the independent variable (pharmacological evaluation of artemether and lumefantrine), capillary sampling (200 µl) and venous sampling was done between 0.5-8 hours post-administration of the 6^{th} AL dose in the 3-day group and the 5^{th} AL dose in the 5-day group and on days 8, 14, and 21 for all groups. For the dependent variable, microscopy was done and dry blood spots (DBS) for LAMP were collected at days 0, 7, 14, 21, 28, 35 and 42. Since LAMP analysis was performed later, microscopy and clinical symptoms were used to decide treatment outcomes. Treatment outcomes were defined as per the standard WHO classification system: Early Treatment Failure (ETF), Late Parasitological Failure (LPF), Late Clinical Failure (LCF) and Adequate Clinical and Parasitological Response (ACPR). (**Supp. Fig. 3)** Early or late clinical failures within 14 days were considered as recrudescent parasites and were treated with quinine. Clinical failures between days 15-42 were treated as a new episode of malaria. AL was re-prescribed for these new episodes and, if eligible and consenting, these children were re-enrolled in the study.

Our sub-study to understand the length of uRDT positivity utilized the data collected from the EXALT parent study. In addition to the parasite density on day 0 and every 7 days thereafter

from the EXALT study, we administered a uRDT at each time point. While DBS were taken at each time point, this study did not perform LAMP and instead used the microscopical evaluation as the gold standard for diagnosis of malaria and treatment outcomes.

Human subjects research

Parents/guardians made the decision to enroll their child in the study. Study physicians were responsible for the informed consent discussion in the clinic. All conversations were done in the appropriate language for the adult parent/guardian and both written and verbal consent was required. If the parent/guardian was unable to read or write, their fingerprint was taken as a substitute for their signature. For blood specimens that were collected from children, permission for specimen banking and future use of biological specimens was also included in the consent process.

Once verbal and written consent were attained, a series of evaluations such as a detailed history, physical examination and measurements of temperature, height and weight were taken. Blood was then collected for hemoglobin testing and malaria thick and thin smear to decide if the participant was eligible for study enrollment. If enrolled, parents/guardians were instructed to return on a specified follow-up day; if they failed to return on that day, a home visitor was sent to encourage them to return to the clinic (active follow-up).

Other considerations were that all HIV-infected children were maintained on anti-retroviral therapy (ART) throughout the course of the study. Moreover, parents were allowed to withdraw

their children from the study at any time and anonymity of participants was preserved even during analysis.

Clinical and laboratory evaluations

Microscopy

Thick smear blood slides were obtained from capillary finger-pricks and parasite density was counted by the laboratory technicians at the time of presentation. The density was estimated by counting the number of asexual parasites per 200 leukocytes, assuming a leukocyte count of 8000/μl (or per 500 leukocytes, if the count is <10 asexual parasites/200 leukocytes). Smears were considered negative if examination of 100 high-powered fields did not reveal parasites. If the thick blood smear was positive, the patient was diagnosed with malaria. If the smear was negative, the patient was managed by medical officers at their usual clinic. All subjects positive for *P. falciparum* and with an uncomplicated case were referred to the study medical officers to determine further eligibility for study enrollment. Double readings on all smears were done to ensure accuracy and any discrepancies were resolved by the head laboratory technician.

Rapid diagnostic test (RDT)

At every time point, including at enrollment (time=0), a microscopic slide was read and an Alere™ Ultra-Sensitive Rapid Diagnostic Test (Reference number: 05FK140, Test lot number: 05LDB001A, Sub: A, Diluent lot number: 05BDDA145, Manufacture date: January 2018 Expiry date: December 2019) was performed. Assay diluent was then added and the uRDT was left for

20 minutes before being read. **(Supp. Fig. 2)** RDT results were read only once and recorded as a binary variable

Loop-mediated isothermal amplification (LAMP)

LAMP Assays were performed using Loopamp™ MALARIA Pan/Pf Detection Kit (Eiken Chemical Company, Japan) (41). A DBS of finger-prick blood was also made on cellulose filter paper at each time point (Whatman™ 1001-090 Grade 1 Qualitative Filter Paper, Diameter: 9cm, Pore Size: 11μl). All DBS were kept at 4 °C in our laboratory in Tororo, Uganda. Although Uganda has the capacity to perform LAMP analysis, all samples were analyzed at the Yale School of Public Health, Laboratory of Infectious Diseases, New Haven, Connecticut, 4 months later. DBSs were analyzed in batches of 25 and DNA was extracted using reagents included in the LAMP kits. The tubes, along with positive and negative controls, were placed into the LF-160 LAMP incubator (Eiken) with a maximum of 16 tubes at one time. All samples were incubated for 40 minutes at 65 °C, then at 80 °C for 5 minutes. Results were read by a single individual observed for florescence by placing each tube under a UV light.

Data management and statistical analysis

All data was collected on standardized case record forms (CRFs) by study physicians. Lab results were recorded first in the laboratory record book and then transferred over to the CRFs. Study coordinators reviewed the CRFs regularly for completeness and accuracy. Once all forms were complete, data was double entered onto a computerized database to verify accuracy. Data was then archived to a large-scale digital tape daily and once a month, a complete backup of the

tape was transported off-site to the Kampala Data Management Center (DMC) where it was uploaded to an online secure server for read-only access.

From the secure server, data was downloaded as a Microsoft Access database and then exported to Microsoft Excel for cleaning. Subjects with one or more missing data point (for parasite density or uRDT outcome) were removed and excluded from the analysis. In Excel, the proportion of all uRDTs that were positive at each time point, for ACPR children only, were plotted with an exponential line of best fit. This graph was then stratified based on HIV status and AL therapy assigned. Next, data was imported into SPSS (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.) where a Mann-Whitney independent t-test was run to decipher if HIV status or AL regimen impacted the length of uRDT positivity. To confirm which variable was correlated with time to uRDT negativity, a linear regression was run with parasite density, age, HIV status and duration of AL therapy as independent variables and time to uRDT negativity as the dependent variable. Lastly, a chi-square analysis was performed to study the agreement between microscopy and uRDT outcome as well as the ability of the uRDT to predict a TF.

Results

Patient demographics

Between 21st of February 2018 and 12th of December 2018, 305 children were screened at MGH hospital. Of these children, 26.6% met the inclusion criteria (81 children). **(Fig. 2)** The most common reasons for exclusion included the following: negative blood smear, mixed infection with non-falciparum species, complicated/severe case of malaria, prior anti-malarial use, lack of access to uRDT at the time of enrollment and decline of study participation. The mean age of the study participants was 6 months (Range: 2 months to 16 months). Twenty children were HIV positive and 61 were HIV negative. Lastly, the sample was split with 57% of all children on a 3-day AL regimen and 43% on a 5-day AL regimen.

Treatment outcomes of enrolled participants

Initial parasite density of enrolled participants ranged from 64 parasites/µl to 205360 parasites/µl with an average of 41520 parasites/µl. **(Fig. 2)** Of the 81 enrolled children, n=26 experienced an adequate clinical and parasitological response (ACPR), n=21 experienced a late clinical failure (LCF) with fever and a positive smear between days 4-42 post-treatment, and n=34 experienced a late parasitological failure (LPF). Overall, 67.9% of all children experienced a TF.

Persistence of positive uRDT after treatment with AL

To analyze the length of uRDT positivity following treatment for microscopically-confirmed malaria infection, only individuals who experienced an ACPR were considered. Children with LPF or LCF, by definition, presented with parasites detected by microscopy during follow-up

and therefore were excluded from this analysis to avoid impacting the overall line of best fit. Of the 26 children with an ACPR, only 21 children had a uRDT result at every time point during the 42-day follow-up period. The remaining 5 children, who had one or more missing uRDT result during the 42-day period, were excluded from the analysis.

Initially, the proportion of uRDTs that were still positive at each time point was graphed. Data points from all 21 ACPR children were included in this curve regardless of whether they were HIV+, HIV-, on a 3-day AL regimen or on a 5-day AL regimen. **(Fig. 3a)**

The mean length of uRDT positivity among these 21 children was 9.2 days with a standard deviation of 11.3 days. Using the best-fit regression line, with an R^2 of 83.3%, it can be seen that approximately 50% of all uRDTs will remain positive for 11 days post treatment of malaria. Similarly, 21 days post effective treatment of malaria, 25% of all uRDTs will still be positive. A visual portrayal of these results for each participant is shown in **Figure 3b**. Six individuals (ID: 50082, 50069, 50107, 50086, 50109, 50134) had a positive uRDT result after a negative uRDT result was recorded at least 7 days prior even though their microscopy result was negative at both points.

Duration of uRDT positivity based on antimalarial treatment regimen and HIV status

Using the 21 children described above, we plotted the proportion of all uRDTs that were positive at each time based on regimen of AL therapy that they received (3-days or 5-days) and their HIV status (positive or negative). Our sample had 10 participants who were on a 3-day AL regimen and 11 participants who were on a 5-day regimen. A regression line of best fit to approximate the

two sets of data points had an R^2 value of 92.2% for the children on AL for 3 days and 76.6% for children on AL for 5 days. A simple extrapolation showed that 50% of children still had a positive uRDT on day 8 for those on 5-day AL therapy as opposed to day 14 for those on 3-day AL therapy. **(Fig. 4)** A visual representation of the uRDT outcome at each time point for each participant stratified by AL regimen is shown in **Supplementary Figure 4**. A Mann-Whitney t-test was done to compare the mean time of uRDT positivity of the 3-day and 5-day group. No significant difference was found in the length of uRDT positivity ($p = 0.416$). This association was later corroborated in the linear regression model ($p = 0.877$). **(Fig. 6)**

Similarly, the analysis described above was repeated on 5 HIV+ and 16 HIV- children. **(Fig. 5 & Supp. Fig. 5)** Half of all uRDTs still returned a positive outcome on day 6 for HIV- children and day 13 for HIV+ children using the approximate trend line. This difference in uRDT length of positivity between HIV positive and negative children was not significant ($p = 0.655$ from t-test and $p = 0.715$ from linear regression model). **(Fig. 6)**

Additional analysis using a linear regression model also suggested that duration of AL therapy or HIV status were not significantly associated with a possibility of treatment failure by day 42. **(Fig. 7)** Treatment failure was classified as an LCF or an LPF by day 42. The p value for the AL regimen was 0.276 and that for HIV status was 0.779. This analysis was repeated with a chi-square crosstabulation and the same non-significant association for AL therapy, HIV status and treatment outcome was seen. **(Supp. Fig. 6 & 7)**

Parasite density and length of uRDT positivity

Using all the 21 children with a uRDT at all time points, we performed a linear regression analysis to determine the correlation between parasite density at day 0 and length of uRDT positivity. **(Fig. 6)** Age, HIV status and duration of AL therapy were included in the analysis as potential confounders and were added individually to the regression model.

A significant association was found between initial parasite density how long it takes for the uRDT to turn negative ($p = 0.006$). Since parasite density and uRDT length of positivity were both modelled as continuous variables, the standardized beta coefficient suggests that an increase in parasite density of 1 parasite/μl results in 0.6 additional days of uRDT positivity. Of all confounders, age was found to have the greatest impact on the beta coefficient reducing it from 3.807 to 3.381. Another linear regression model found no significant correlation between initial parasite density and the likelihood of TF ($p = 0.402$). **(Fig. 7)**

Microscopy and uRDT concordance

Concordance between microscopy and uRDT was assessed using a chi-square test of independence. Data from all time points where both a parasite density measure and an uRDT result were available were included in this analysis. Parasite density was converted into a binary categorical variable. **(Fig. 8)**

Of the 4 possible combinations (uRDT positive/parasites present, uRDT positive/parasites absent, uRDT negative/parasites present and uRDT negative/parasites absent) the parasite density and uRDT outcome matched 67.5% of the time (320/474). The true positive rate

(sensitivity) was calculated to be 91.3% and the true negative rate (specificity) was 55.3%. Twenty nine percent of the time, the uRDT was positive but the smear was negative (n=140), and the reverse was true only 0.03% of the time (n=14). We were able to reject the null hypothesis of independence and conclude that the parasite density and uRDT outcome are significantly correlated with one another ($p = 8.71e^{-23}$). In other words, the odds of the uRDT being positive given that microscopy also detects parasites is 12.98 (CI: 7.18 - 23.45).

uRDT as a predictor of treatment failure

Due to the uRDT's highly sensitive nature and ability to detect parasitemia at lower limits of detection, we hypothesized that the uRDT could be used to indicate a possible treatment failure (LCF or LPF), by becoming positive prior to microscopy. Our initial sample consisted of 21 LCF and 34 LPF participants. However, after excluding subjects with missing data, 17 LCF and 33 LPF were left. Treatment failure (TF) was detected at various point throughout the 42-day period with 1 participant failing on day 14, 19 participants failing on day 21, 14 participants failing on day 28, 7 participants failing on day 35 and 9 participants failing on day 42.

Regardless of when the treatment failure was recorded (as determined by microscopy +/- fever), we compiled all participants in a table with the day of failure denoted as "Day F" **(Fig. 9)** The uRDT outcome on day F was portrayed alongside the uRDT outcome 7 days prior to when TF was designated (F-7) and 14 days prior to when TF was designated (F-14). Of all TFs, irrespective of the day, 54% had a positive uRDT only 7 days before TF and 34% had a positive uRDT both 7 days and 14 days prior to TF. To test the significance of this association, we performed a Fisher's exact test to account for the small cell sizes. **(Fig. 10)** A two-sided test

suggested that the uRDT on day F and the uRDT on day F-7 were not independent and instead significantly associated with one another ($p = 0.015$). Repeating the Fisher's analysis for the relationship between uRDT on day F and day F-14 was not significant at an alpha of 5%.

Discussion

Alere's™ ultra-sensitive RDT has revolutionized malaria detection particularly in low transmission and elimination settings. However, its increased sensitivity comes with challenges in field settings, such as false positives relative to microscopy, a direct result of its continued positivity following a malaria infection.

A recent literature review on the persistence of positivity of RDTs after anti-malaria treatment, by Dalrymple et al., showed that when using an HRP2-RDT, 50% of patients present a negative test by day 19 (CI: 10-31) when an ACT is given on day 0. (3) Our findings on the uRDT suggest that it takes 11 days, well within the 95% confidence interval. Similarly, a prospective cohort study of 32 pregnant women found that 41% of women still returned a positive HRP2-RDT at day 7 after microscopy+RDT diagnosis and prescription of an ACT. (42) In some instances, *Pf*HRP2-RDTs have shown a positive result more than 5 weeks after antimalarial treatment (with parasite clearance confirmed by microscopy). (43) Studies on post-treatment HRP2 degradation suggest a first-order exponential decline with a median half-life between 3.0-4.7 days in different African countries. (44) Our study aligns with all these prior studies and provides additional compelling evidence about one of the biggest challenges of HRP2-RDTs- the

extended period of time that the RDT remains positive even after treatment and elimination of the parasite.

While our study did not incorporate other RDTs, it has been shown that pLDH-RDTs remain positive for significantly less time (50% present as positive between 1-7 days post-treatment). (3) Therefore there may be a benefit to limiting HRP2-RDTs to low transmission settings and pLDH-RDTs to high transmission settings, but this suggestion would need to be tested further. All in all, any RDT test result must be confirmed with either microscopy or PCR to account for antigen persistence, a recommendation in line with current WHO guidelines.

We hypothesize that the 6 individuals described earlier, who were ACPR and that had a positive uRDT result after a negative uRDT result, likely arose due to simple random error. Three out of the six individuals had the positive uRDT on day 35 or 42. Therefore, the other possibility is that the uRDT was picking up a treatment failure that was imminent. Since our follow-up period ended on day 42, the only way to confirm this hypothesis is by performing LAMP tests at those time points.

No significant difference was seen in length of uRDT positivity between HIV positive and negative individuals. However, this effect may have been dampened by the small sample size of HIV positive children in our study. In fact, for HIV+ children, it took an additional 7 days before 50% of all the tests were no longer positive. HIV has been shown to directly and adversely impact the response to malaria. (45) Due to their impaired immune system, HIV+ individuals produce lower levels of interferon γ and tumor necrosis factor in response to malaria parasites.

(45) This could lead to higher parasite burdens, longer circulation of the HRP2 antigen and longer positivity of the uRDT. Similarly, while we did not see a significant association between course of AL therapy and length of positivity, this may have also been due to a small sample size. Theoretically, however, this was expected. Multiple studies have confirmed that the parasite clearance half-life ($PC_{1/2}$) is 1.8 to 3.0 hours for a 3-day artesunate therapy. (46) Taking a longer treatment course is unlikely to impact how long parasites remain in the body and the HRP2 antigen presence.

Participants who enrolled in the study with higher parasitemia were found to have longer persistence of the HRP2 antigen, a finding also reported previously. (47) We hypothesize this is because of a higher proportion of remaining parasites following initial AL treatment however PCR analysis would have to confirm this. Interestingly, a recent study suggested that artesunate clears non-ring stage parasites by a process known as "pitting" where the parasite is expelled, the erythrocyte is resealed and then returned into circulation. (48) Additionally, the HRP2 antigen, that is present in the cytosol of the erythrocyte soon after parasite invasion, continues to be expressed on the erythrocyte membrane even after the parasite is expelled. (48) Thus, a higher initial parasitemia is likely to lead to more pitted and HRP2 covered erythrocytes. These revived erythrocytes have a reduced lifespan of 1-4 weeks which would explain why the uRDT remains positive for an extended period of time post-treatment.

In the same study by Dalrymple et al. cited earlier, children and those subjects on artemisinin combination therapies (ACTs) demonstrated greater persistence of the HRP2 antigen through longer RDT positivity compared to adults or subjects on other antimalarials (artemisinin

monotherapy, chloroquine, quinine, primaquine, sulfadoxine-pyrimethamine and mefloquine) (3). While we did not look at the effect of other antimalarials, age was shown to be the strongest confounder in our study. In our linear regression model, age had a standardized beta coefficient of -0.226 suggesting that every increase in age by 1 year would result in a decrease in the time to uRDT negativity by 0.226 days. Although this association was not significant, it follows the same direction as reported in previous studies. One plausible reason behind this relationship is that children who are younger are less likely to have developed an acquired immunity through repeated malaria infections. (49, 50)

We expected a longer course of AL therapy to prevent recrudescence of the malaria parasite or prevent a new infection. This is because artemisinin and its derivatives have a plasma half-life of <3 hours and its partner drugs, lumefantrine, has a half-life of 5 days. (51) It was no surprise to find that 98% of all TFs occurred on day 21 and beyond, when the drug concentrations are below a potent concentration. However, we did not see a reduction in TF due to a longer AL regimen as anticipated. Although we were only looking at the association between the outcome on day 42, the pharmacodynamic results from the EXALT study will shed definitive light on the impact of a longer AL regimen.

Even though a significant association between microscopy and the uRDT was found, 29.54% of the time the uRDT was positive but no parasites were detected by microscopy. This is likely due to the circulating antigen post treatment. More concerning however are the 14 individuals who were positive by microscopy but negative by uRDT (0.03%). While this may be a simple microscopy error (5 of these individuals had a parasite density of <100), an unpublished

household survey from Uganda has suggested that the prevalence of HRP2 deletions is 1.6% (25/1493). (25) Genomic analysis will need to be performed to determine if these 14 individuals had the HRP2 deletion. This loss of the *pfhrp2* gene is a cause for concern as HRP2-RDTs become ubiquitous in malaria diagnosis.

Early detection of a TF is crucial for the quick and timely switch to an alternate second-line anti-malarial thus avoiding any severe complications. The day 3 HRP2 concentration has been shown to indicate eventual recrudescence in individuals post antimalaria treatment. (44) Along the same lines, in this study, we report a novel ability of the uRDT to predict an upcoming TF 7 days prior. A study in 2001 also tested the ability of a *Pf*HRP2 dipstick on day 7 and day 14 to predict TF but found no association. (47, 52) Alere's more sensitive RDT, compared to its predecessors, may allow us to reliably anticipate a TF. More research must be done on a larger scale to validate the accuracy of the uRDT as a TF predictor.

However, we must consider the practical implications of using an RDT as an early signal of TF. Not only is it infeasible to require individuals to return following AL treatment to test for a possible TF, there is also the likelihood that the uRDT is positive due to persistence of antigen and not recrudescence of the parasite. Therefore, we do not recommend implementing the uRDT on a large scale as an early indicator of TF. Instead, the uRDT should remain for its intended purpose- as a more sensitive test to eliminate and eradicate malaria in low transmission settings.

Conclusion and limitations

One of the major limitations of this study is the small sample size used for the analysis. While the statistical tests account for the small sample size, additional studies need to be done to support the associations suggested in this study. One of our key hypotheses was that AL regimen would be associated with treatment outcome. We saw an incidence of treatment failure in 71.7% of participants in our 3-day AL group and 66.7% in our 5-day group. Using this as a baseline incidence, to achieve a power of 70% at an alpha of 0.05, we would ideally need >1000 children in each treatment group. A similar sample size would be needed to understand if HIV status affects treatment outcome.

Another limitation was that initial diagnosis of malaria was done with microscopy and not uRDT. This could have limited our study to only those individuals who had a high enough parasitemia to be microscopically detected. However, it is unlikely that not including low parasitemia cases in our study would have impacted our results.

Finally, this study used microscopy as the gold standard for malaria detection and diagnosis. Since LAMP is considered a far more sensitive and accurate measure of malaria parasite presence, future studies should consider incorporating LAMP into the analysis and potentially as the gold standard. It would also be interesting to see if LAMP's ability to predict TF is comparable to that of the uRDT.

Overall, the evidence from this study highlight the potential and drawbacks of Alere's new uRDT. Its highly sensitive nature means the test is likely to return a positive result long after the clearance of malaria parasites. Ideally, if the uRDT is used to establish a malaria diagnosis, physicians should examine prior medical records and take into consideration that the uRDT may still be positive from a previous malaria infection. Concern over false positive and incorrect diagnoses recommend that the use of Alere's uRDT should be limited to low transmission settings.

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