Predictive parasite density based on a linear regression model obtained from the time of positivity of two malaria rapid diagnostic tests (SD Bioline Malaria Ag-Pf/Pan and Paracheck™-Pf) in Côte d'Ivoire

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Rapid diagnostic tests (RDTs) are the current complement to microscopy for ensuring prompt malaria treatment. However, non-estimation of the parasite density is one of the limits of these RDTs. This study aimed to estimate the parasite density based on the time of positivity of two RDTs, SD Bioline Malaria Ag-Pf/Pan and Paracheck™-Pf. A highly parasitized blood sample underwent successive dilutions. On each resulting dilution, a thick blood film and two RDTs were performed. The time of onset of the positivity of the diluted blood samples was recorded from each RDT. A linear regression model was determined to estimate a range of parasite density from the time of positivity. The model was then assessed using known parasites density of additional blood samples from symptomatic malaria patients. For each RDT, the curve representing the time of positivity showed three stages. The latency stage, before the test line of the RDT appears, lasted 2 min 40 s for SD Bioline and 3 min 14 s for Paracheck. The optimal stage during which the line of positivity appears lasted from 2 min 40 s to 6 min (SD Bioline), and from 3 min 14 s to 7 min 2 s (Paracheck). A phase of pre-negativity spanned from 6 min to 14 min 20 s (SD Bioline) and 7 min 2 s to 14 min 40 s (Paracheck). Two linear regressions equations were drawn to link the mean time of positivity appearance to the mean parasite density (Dm), that is, $Dm = -119.5t_m + 81943.3$ (SD Bioline) and $Dm = -142.0t_m + 99779.1$ (Paracheck). Based on the statistical models, RDTs time of positivity ranged from 2 min 40 s to 11 min 25 s, and from 3 min 14 s to 11 min 33 s for SD Bioline and Paracheck respectively. The linear regression models obtained from the time of positivity of RDTs allows the prediction of malaria parasite density. There was no significant difference between the theoretical malaria parasite density and the one observed with microscopy.

Key words: Malaria parasite density, linear regression model, rapid diagnostic tests (RDTs), Côte d'Ivoire.
unnecessary treatment, the world health organization (WHO) recommends parasitological confirmation of malaria whenever possible, by Rapid Diagnostic Tests (RDTs) or by microscopy (Feachem et al., 2010; Zarocostas, 2010).

Existing tools for the diagnosis of malaria include light microscopy, RDTs and nucleic acid detection test (Chiodini, 2014; Bronzan et al., 2008). Microscopic detection of parasites on Giemsa-stained blood smears has been the mainstay of malaria diagnosis in laboratories for more than a century (Wongsrichanalai et al., 2007). However, it requires highly qualified microscopists and reliable equipment, which are often lacking in remote areas where malaria is more prevalent (Moody, 2002). For over a decade, the development of RDTs has enabled reliable diagnostic testing in situations where previously only clinical diagnosis was available (Bell et al., 2006). The availability RDTs constitutes an opportunity for parasite-based malaria diagnosis in rural African settings beyond the reach of microscopy services (Masanja et al., 2012).

Although the good performance of malaria RDTs has been established, the functioning and accuracy of RDTs in the fields can be affected by several factors, including manufacturing defects, storage, transport, and end-user performance (WHO, 2003). In addition, the lack of estimation of parasite density constitutes one of the limitations of the RDTs which positivity relies on the appearance of a band test, the latter related to the parasite density (Makler, 1998). This situation often results in a low coverage of RDT implementation and poor adherence to RDTs outcomes (as the basis for treatment decisions) in clinical practice.

The main objective of this study was to determine a linear regression model based on the time of positivity of RDTs and the corresponding parasite densities.

MATERIALS AND METHODS

Study site

Blood samples were collected in the health center of Anonkouakouté which is located in the district of Abobo, in the North of Abidjan, in Côte d’Ivoire. Abobo is an area where the transmission of Plasmodium falciparum is intense and perennial. The laboratory procedures were carried out at the Malarialogy Department of Institut Pasteur of Côte d’Ivoire.

Selection of technicians

Two lab technicians involved in the study were selected based on their previous training, all of whom were re-trained to diagnose malaria in the laboratory according to standard operational procedures (WHO, 1991). The technicians were also trained to perform and read Paracheck and SD Bioline RDTs. A third lab technician was designated to analyze all the discrepant slides.

Preparation of dilutions

Successive two-fold dilutions were carried out from one highly parasitized field blood sample (154,378 asexual parasites/µL of blood). To do that, two hundred microliters of a malaria infected blood sample was first transferred into a microtube containing an equal volume of uninfected O+ blood. Then, 200 µL of this first dilution were transferred into tubes containing an equal volume of uninfected O+ blood. Serial dilution was made under the same conditions until dilution of 1/2048 (10 times). It was noticed that parasite densities decreased from 154,378 to 266 asexual parasites/µL of blood (at ninth dilution).

Thick and thin blood films

Duplicate thick and thin smears were made on the same slide with initial sample used for dilution and the field samples. Duplicate Thick films were prepared from each diluted sample. The first slide was read, while the second slide was archived for quality control purposes. Thick and thin blood smears were stained with 10% Giemsa at pH 7.2 for 30 min. Parasite density was determined based on a putative count of 8000 leukocytes per microliter of blood by reading the thick blood smear and counting the number of asexual parasites against the number of leukocytes using a hand tally counter for 200 high-powered fields. Slides were considered negative if no parasite was detected after reading 200 high-powered fields. Presence of gametocytes was also recorded. Thin blood smears were reviewed for non-falciparum infections. Two microscopists read the slides independently and parasite densities were calculated by averaging the two counts. Readings with discordant results, that is difference in species diagnosis, in parasite density count of 25%, were re-examined by an independent third microscopist. The parasite density was calculated by averaging the two closest densities while the final parasite species was determined by the two concordant reads.

Malaria rapid diagnostic tests

A formally trained lab scientist in performing and interpreting RDTs performed the RDTs and subsequent outcomes were read and interpreted within 15 min according to the manufacturer’s instructions. RDTs were stored correctly that is, in an air-conditioned room with regular temperature monitoring according to the WHO recommendations (WHO, 2003). Two RDTs were carried out on air-conditioned laboratory at the Malarialogy Department of Insitut Pasteur of Côte d’Ivoire.

Briefly, for malaria diagnosis using the Paracheck device, 5 µL of blood sample measured by calibrated pipette was put into the corresponding blood well and six drops of the clearing buffer solution was added as per the manufacturer’s recommendations. The presence of parasites was confirmed after 15 min when two red bands, one as the control “C” and other as the test “T”, were observed. Negative results had only the control band. Results were reported as invalid when no control band appeared after the test.
was repeated twice. The SD Bioline Malaria Ag P.f./Pan 05FK60 rapid test was the one used in the current study. It is a rapid three-band lateral flow chromatographic immunoassay for the qualitative detection of P. falciparum specific HRP2 antigens and/or Pan malarial LDH found in P. falciparum, Plasmodium vivax, Plasmodium ovale and Plasmodium malariae. The test distinguishes between a P. falciparum infection and an infection due to either another plasmodium malaria parasites or a mixed infection in a cassette format. The RDT was carried out with 5 μL of blood sample. A negative result was indicated by the presence of a single line, the ‘C’ control line, in the result window. A P. falciparum-positive result was indicated by the presence of a colored band in the P. falciparum test line and the ‘C’ control line. The presence of the “Pan” test line and the “C” control line (again, two colored bands) indicated a positive result due to either P. ovale, P. malariae, or a mixed non-falciparum infection. The presence of the “Pan” test and the “C” control lines indicates a P. falciparum positive result or a mixed infection. In case the control line did not appear, the test was considered as invalid and it was repeated immediately. Each RDT test was performed in triplicate by one technician. The time of RDTs’ positivity was recorded using a double entry stopwatch. RDTs (SD Bioline and Paracheck) were performed with initial sample used for dilution, each diluted sample and the field samples.

Linear regression modeling

For each diluted sample, two thick films were prepared and RDTs were performed as indicated earlier. The times of positivity were recorded thrice for each dilution, accordingly. The resulted mean parasite density and the average time of positivity per RDT were used in linear regression modeling. The parasite density was set as the dependent variable, and time of positivity as the independent one. The linear regression model processed with XLstatPro 2013.2 (Addinsoft Paris, France) statistical software for each RDT and included a coefficient of determination (R²).

Assessment of parasite density of field samples from the linear regression model

Parasite densities were predicted using the linear regression models. A total of 15 P. falciparum malaria samples known to be positive by microscopy (Thick and thin smears) were collected into EDTA tubes from symptomatic patients. Inclusion criteria were the following: six months old or more; P. falciparum mono-infection; axillary temperature equal or over 37.5°C at the time of enrolment or history of fever during the preceding 24 h; no evidence of concomitant febrile illness; provision of written informed consent by the participant or by a parent or guardian (for children). Exclusion criteria were as follows: symptoms and/or signs of severe malaria; any danger sign (persistent vomiting; inability to sit, stand, drink or breastfeed); haemoglobin concentration ≤ 10 mg/dl; use of antimalarial drugs during the previous 7 days; use of concomitant medications that may induce haemolysis or haemolytic anaemia from the world health organization (WHO) list of essential drugs. RDTs were performed and times of positivity were recorded for these samples. A theoretical mean parasite density was calculated by each of the linear regression model given the mean time of positivity recorded previously. In order to assess degrees of concordance, the theoretical parasite density was compared to the observed parasitemia determined from the slides microscopy readings.

Ethical considerations

The study was approved by the Comite National d’Ethique de la Recherche of Côte d’Ivoire. After information and appropriate explanations, adult participants, parents or legal guardians of all children willing to participate in the study gave their written consent before sampling.

The RDTs were run free of charge and patients were treated by physicians.

Statistical analyses

The data were analyzed with the software XLstatPro 2013.2 (Addinsoft Paris, France) an add-on to the Excel 2007 application. The linear regression equations resulted from a Pearson test of linear regression. To compare calculated to observed densities, the Test of Wilcoxon /two-tailed test was performed. The statistical significance threshold was 5%.

RESULTS

Time to positivity of SD bioline malaria-AgPf/Pan and Paracheck-Pf RDTs

Figures 1 and 2, shows the variation of the average time of positivity of RDTs (SD Bioline and Paracheck) from diluted sample. The curve obtained has three stages. The first one corresponded to the phase of migration, that is, it indicates the minimum time spent before the appearance of positivity line. This time ranged from 0 to 160 s (0 to 2 min 40 s) and 0 to 194 s (0 to 3 min 14 s) for SD Bioline and Paracheck, respectively.

The second stage is the range of time corresponding to the appearance of positivity. For SD Bioline, it was 160 to 360 s (that is, 3 to 6 min) and 194 to 422 s (that is, 3 min 14 s to 7 min 02 s) for Paracheck. The third stage is related to the time of pre-negativity beyond which the results are to be considered not accurate. It spans from 360 to 860 s (that is, 6 to 14 min 20 s) for SD Bioline and 422 to 880 s (that is, 7 min 02 s to 14 min 40 s) for Paracheck.

Theoretical models to determine parasite densities

Figures 3 and 4, shows the theoretical models of linear regression obtained from the dilutions of an initial highly parasitized sample. For SD Bioline (Figure 3), the model and the coefficient of determination R² are: Dm = -119.5 tm + 81943.3; and R² = 0.371. As per the Paracheck test (Figure 4), they are respectively: Dm = -142.0 tm + 99779.1; and R² = 0.307. Two types of time range were highlighted:

For the first type of time range, there is a good concordance between a given time of onset of positivity and the parasite density. This time ranged from 2 min 40 s to 11 min 25 s for SD Bioline; and 3 min 14 s to 11 min 33 for Paracheck. Beyond these periods of time, data are no longer accurate.
Table 1. Theoretical estimation compared to observed parasites density SD bioline malaria AgPf/Pan.

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<th>Sample ID</th>
<th>Mean time of positivity (s)</th>
<th>Obs. mean PD (Tpz/µl)</th>
<th>Pred. mean PD (Tpz/µl)</th>
<th>95% CI of mean Pred. PD</th>
<th>SD pred. mean PD</th>
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PD: Parasite density; Obs and Pred: observed and predictive; CI: confident interval; SD: standard deviation.

Table 2. Theoretical estimation compared to observed parasites density for Paracheck™ - Pf.

<table>
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<tr>
<th>Sample ID</th>
<th>Mean time of positivity (s)</th>
<th>Obs. mean PD (Tpz/µl)</th>
<th>Pred. mean PD (Tpz/µl)</th>
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PD: Parasite density; Obs and Pred: observed and Predictive; CI: confident interval; SD: standard deviation.

Theoretical estimates of parasites density compared to observed

Tables 1 and 2 show theoretical parasite densities calculated from the linear regression models of the two RDTs and its comparison with parasite densities obtained from microscopy slides readings. When the average time of onset of positivity increased, the calculated parasite density decreased. The average time of appearance of the test line ranged from 190 to 472 s, with a corresponding calculated malaria parasite density ranging from 59 234 to 25 529 asexual parasites/µl for SD Bioline (Table 1). With the Paracheck test, the average time of onset of positivity ranged from 139 to 575 s, with a corresponding calculated malaria parasite density ranging from 76 558 to 11 012 asexual parasites/µl (Table 2). There was no significant difference between malaria parasite density obtained from microscopy, and the calculated ones obtained by the linear regression model (p = 0. 0589 for SD Bioline; p = 0. 094 for Paracheck).

DISCUSSION

Recently in many malaria endemic countries, the use of RDT as a fast and easier method of malaria diagnosis
Figure 1. Variation of the mean parasite density of diluted samples according to the mean time of positivity obtained SD Bioline Pf/Pan RDT.

Figure 2. Variation of the mean parasite density of diluted samples according to mean time of positivity obtained with Paracheck-Pf RDT.
Figure 3. Linear regression model linking theoretical mean parasites densities to successive mean time of appearance of the line of positivity from the SD Bioline Pf/Pan outcome. Note: The negative slope is indicative of a negative correlation between the parasite density and the time of appearance of the positivity line displayed with SD Bioline Pf/Pan RDT. Equation of the linear regression: \( D_m = -119.5 t_m + 81943.3 \). Coefficient of determination: \( R^2 = 0.307 \).

Figure 4. Linear regression model linking theoretical mean parasites densities to successive mean time of appearance of the line of positivity from the Paracheck Pf outcome. Note: The negative slope is indicative of a negative correlation between the parasite density and the time of appearance of the positivity line displayed with Paracheck RDT. Equation of the linear regression: \( D_m = -142.0 t_m + 99779.1 \). Coefficient of determination: \( R^2 = 0.371 \).
has been promoted to replace the tedious and time-consuming microscopic methods (WHO, 2012). From an initial positive blood sample, this study derived nine samples by dilution of which the average parasitemia ranged from 154 to 378 asexual parasites/µl of blood. Whatever the parasitemia level, there is a latency time before formation of positive band that lasted less than 2 min 40 s for SD Bioline, and 3 min 14 s for Paracheck. The phase of occurrence of the positivity line appeared between 2 min 40 s and 6 min for SD Bioline, and 3 min 14 s to 7 min 02 s for Paracheck. Within these ranges of time the corresponding parasites densities were 154 378 to 1450 asexual parasites/µl of blood for both tests. At parasitemia densities below 1450 asexual parasites/µl of blood the corresponding time to positivity for SD Bioline and Paracheck was 6 min to 14 min 20 s and 7 min 02 s to 14 min 40 s, respectively. This observation is similar to what was reported by Gaye et al. (1998) showing that band intensities were correlated with high parasitemia (164 160 to 40 880 asexual parasites/µl of blood) for about 5 min of optimum appearance.

The time of latency of the two RDTs could result from the time spent by the sample to migrate from the sample well to the "T" test line. The optimum phases of positivity could be explained by high parasitaemia. Pre-negativity stage could be due to low parasite density and the sensitivity of the RDTs. Factors that affect the sensitivity and specificity of RDTs, such as low parasitemia, are main challenges for malaria diagnosis. Previous studies estimated that parasitaemia less than 500 asexual parasites/µl of blood, that is, positive microscopy slides, gave negative results with RDTs (Djalle et al., 2014; Shakya et al., 2012). It was suggested that lower levels of HRP2 during a malaria episode with low parasitaemia might not be detected by RDTs (Desakorn et al., 2005) because malaria antigenaemia depends on the parasite biomass in the patient’s body during an acute episode (Dondorp et al., 2005). Furthermore, deletions or mutations in the HRP2 gene may reduce the sensitivity of these RDTs (Kumar et al., 2013; Wurtz et al., 2013; Maltha et al., 2012). Other factors, such as the ‘prozone effect’ for Paracheck at high parasite densities (Gillet et al., 2009) and the presence of anti-HRP 2 in humans (Biswas et al., 2005), might explain why some tests give negative results despite significant parasitaemia.

The negative slopes observed for the two RDTs show that both parasite density and time of onset of the lines could vary in an opposite direction. The higher the parasite density, the shorter the time of appearance of the T lines. Beyond the time points of 11 min 25 s (SD Bioline) and 11 min 33 s (Paracheck), the results given by the respective RDTs are no longer accurate. Intervals for results accuracy are 2 min 40 s to 11 min 25 s for SD Bioline and 3 min 14 s to 11 min 33 s for Paracheck and are consistent with the corresponding mean parasite densities for the two RDTs. The coefficients of determination, $R^2 = 0.371$ for SD Bioline, and $R^2 = 0.307$ for Paracheck indicate that 37.1 and 30.7% of the variability of the average time of appearance of the band could be explained by the variation in parasite density.

This study show that for the two RDTs, when the average time of appearance of the T line increases, the theoretical mean parasite density decreases. The principle of SD Bioline Malaria Ag-Pf/Pan is to detect living as well as dead parasites circulating in peripheral blood. The more live parasites there is, the shorter the time for the appearance the bands. This principle of operation could explain the negative correlation between the time of appearance of the bands and parasite density. Our results are in agreement with those found by Ashley et al. (2009). They showed that the CareStart RDT test, of the same principle as SD Bioline, can become negative two days after. In contrast, the Paracheck test detects the presence of the HRP2 protein, which could persist in the peripheral blood even after clearance of parasites. Thus, the appearance of the positivity line with Paracheck even in a short time does not necessarily mean a high parasite density.

The HRP2-based RDTs test could bias the results. Persistence of the positivity line for Paracheck in a long run reduces its usefulness in monitoring response to treatment. Swarthout et al. (2007) showed that during follow-up after treatment, 98.2, 94.6, 92 and 73% of children were still false positives according to the Paracheck test on day 14, 21, 28, and 35, respectively. Cases incorrectly identified as positive by the RDTs might also be due to cross-reactivity with human autoantibodies (Bartoloni et al., 1998; Iqbal et al., 2000) and other infectious diseases (Gillet et al., 2013).

However, the main issue with HRP2-based RDTs is the presence of a residual antigenaemia resulting in persistent positive testing outcomes several weeks after a successful treatment (Mayxay et al., 2001; Swarthout et al., 2007). This is of particular concern in moderate/high transmission areas where false positive RDTs may frequently result in provision of anti-malarial treatment to patients who are no longer malaria infected. This phenomenon may also impair health workers’ trust in and adherence to RDTs results (Baiden et al., 2012; Olliaro, 2009).

The linear regression models obtained from the time of positivity of diluted samples allow the prediction of malaria parasite density from sample collected from any symptomatic patient. No significant difference appears between the theoretical malaria parasite density and the ones obtained by microscopy.

A limitation exists since the antigens may persist in the blood of the patient for weeks. Another limitations is variability in the consistency of the papers used in the RDTs. For a better use of this regression model, patient’s history and clinical data may guide the clinicians’ treatment decisions.
Conclusion

Predictive malaria parasite density based on a linear regression models can be estimated but this estimation should be confirmed by microscopic examination for exact counting. Furthermore, an additional study could be helpful for clinicians who would like to know the parasite density without preparing blood smears.

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Conflict of interest

The authors have none to declare.

REFERENCES


