Accuracy of HRP2 RDT (*Malaria Antigen P.f*®) compared to microscopy and PCR for malaria diagnosis in Senegal

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Rapid diagnosis tests (RDTs) allow for the confirmation of malaria diagnosis. In Senegal, RDTs detecting HRP2 have been adopted in 2008 for malaria diagnosis. However, the sustainability of this strategy requires adequate and regular quality control. PCR on DNA extracted in nitrocellulose band of RDTs enable quality control. A RDT (*Malaria Antigen P.f*®) and a thick smear were performed on patients with suspected malaria. DNA was extracted from the nitrocellulose band of RDTs to which a non-specific PCR and a specific PCR were applied. The results of the RDT were compared with those obtained from the thick smear and the PCR to measure sensitivity, specificity as well as positive and negative predictive values. For 81.6% of the 273 patients involved, the thick smear was positive. Rapid diagnosis tests were positive for 85.7% of the patients. Non-specific PCR was positive on 87.9% of RDTs. *Plasmodium falciparum* was found in 99.5% of patients and *Plasmodium ovale* appeared in only 0.4% of patients. Sensitivity of the Malaria Antigen Pf® RDT in relation to thick smear and to PCR was 98.2% and 97.1% respectively. Quality control with PCR on the nitrocellulose band performed several months after it was used confirms its adequate level of sensitivity. The collection and screening of DNA present in already used RDT is a good means of quality control for this tool. It is also a relevant alternative to the molecular approach in the context of a reduction in the transmission of malaria.

Keywords: Malaria, Rapid diagnosis test, PCR

Introduction

In Senegal, adequate treatment of patients is one of the essential components of the strategies developed by the National Malaria Control Programme (NMCP). It is based on a clinical diagnosis, confirmed by a biological diagnosis and followed by the prescription of an effective treatment using ACTs. A NMCP survey showed a low demand of thick smear which is the reference technique.¹ It also indicated that in most peripheral health structures, the quality of field microscopy diagnosis is not good enough, which leads clinicians to treat patients for malaria whatever the results of microscopy. In 2008 therefore, only 70% of inpatients were biologically confirmed by a thick smear. Microscopy was also found to be seldom available in those health structures, especially in the communities where most cases of malaria

occur. To meet that deficit, Senegal chose in 2008 to resort to RDTs for all suspicions of malaria and to condition the starting of an ACT based treatment of malaria on a positive test. The *Malaria Antigen P.f*® test (Standard Diagnostics, Inc, Suwon, Kyonggi-do, Korea) which only detects the HRP2 antigen of *Plasmodium falciparum* was selected. It is now available in all public health structures in the country and also at community level.² However, the acceptability of the RDTs by medical staff, especially in the face of a negative result, remains a major obstacle to a proper use. Indeed, for most medical staff, a negative result is an indication of the low sensitivity of the test (false negative). In the same way, numerous false positives due to the persistence of HRP2 are a source of confusion.

Making the national strategy sustainable will depend on finding answers to these issues. The aim of our study was to measure the sensitivity of the *Malaria Antigen P.f*®RDT, compare it to the thick

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smear and perform a quality control through a PCR on the nitrocellulose band contained in the RDT to determine the presence or absence of plasmodium DNA. This detection technique based on RDTs has been described by several authors^{3,4} and recently the efficiency of DNA extraction using nitrocellulose band of RDTs was demonstrated.⁵

Methodology

We performed a prospective, open study on patients with a suspicion of malaria. The study was carried out in Kasnack health district, in the region of Kaolack. Malaria transmission is seasonal there, from August to September.

The intervention units were health posts in Kasnack health district. Three of the 10 posts of the district were randomly selected and enrolled in the study: Dialègne, Medina Baye and Medina Mbaba. We performed a comprehensive recruitment of all the patients seen in these three health posts during the month of November 2009 for suspected malaria (fever with $T > 37.5^{\circ}\text{C}$ and/or history of fever during the previous 24 hours), clinical signs of likely malaria (headache, nausea, vomiting, arthralgia, dizziness). Informed consent was sought and obtained from all patients enrolled.

Patients with a history of hemorrhagic disease (K avitaminosis, hepatocellular insufficiency, hemophilia) were excluded.

Each of the patients included in the study had a thick smear, a thin smear and a RDT (*Malaria Antigen Pf*[®]). The RDTs were performed based on the recommendations of the manufacturer. Blood smears and RDT were read immediately on the field by the technicians of Kasnack health district. Blood smears were stained with Giemsa and 200 leukocytes were read secondarily to parasitology laboratory of UCAD which is the national referral service by experienced laboratory technicians regularly participating in an external assessment program. Assuming a total leukocyte count of 8000/l, parasite density was determined as the number of asexual parasites $\times 8000/200$. The slide readers were kept blinded to the RDT results.

At the end of the study, all RDTs were taken to the parasitology laboratory and were submitted to a PCR.

We carried out a PCR 12 months after the collection of the samples from all patients involved. DNA was extracted based on the method described by Ndao *et al.*⁶ on the side of the nitrocellulose on which the blood drop is laid. The part where the buffer is deposited is discarded. The rest is cut off and placed in a tube to incubate in 1 ml of bi-distilled water at room temperature. The cutting is done with sterile scissors which have been exposed for 10 seconds to the flame of a bunsen burner. After centrifugation at 9000 rpm (Heraeus Megafuge 16 R, Thermo Scientific, Montreal, Canada) for 30 minutes, the supernatant is disposed of and 200 μl of Chelex 100[®] (Bio-Rad Laboratories Inc Hercules Canada) in a 5% suspension are added to the pellet. All this is then left to incubate for 30 minutes in a water bath at 56°C and then boiled for 8 minutes. The samples are then vortexed for 2 minutes and centrifuged at 9000 rpm for 5 minutes. The supernatant is immediately used for the PCR or conserved at -20°C .

Amplification and detection

A nested PCR to detect *Plasmodium* sp was done using primers described by Snounou *et al.*⁷ (Table 1). The first amplification was done with 10 μl of the extraction product for a total volume of 50 μl . The second amplification was done with 2 μl of the product of the first amplification for a final volume of 20 μl . The DNA was detected by electrophoresis of 10 μl of the product of the second amplification on an agarose gel at 2% with ethidium bromide at 1 mg/l. The photography was made under UV light (Syngène, Frederick, MD, USA). All PCRs were done with a thermocycler (Mycycler Bio-Rad, Canada). A PCR inhibition test was performed on 25% of the negative samples to confirm the results collected.

A specific PCR was carried out on all the samples which were positive to the *Plasmodium* sp PCR based on the first amplification using the primers specific to *P. falciparum*, *P. ovale* and *P. malariae* (Table 1).

Table 1 Characteristics of primer pair described by Snounou *et al.* and Ndao *et al.*

Primers	Sequence nucleotide (5'- 3')	Parasite target	Annealing T ($^{\circ}\text{C}$)	Amplicon name length (bp)
rPLU1	TCAAAGATTAAGCCATGCAAGTGA CCTGTTGTTGCCCTAAACTCC	<i>Plasmodium</i> sp (first amplification)	55	640 rPLU5
rPLU3	TTTTATAAGGATAACTACGGAAAAGCTGT TACCCGTCATAGCCATGTTAGGCCAATACC	<i>Plasmodium</i> sp (second amplification)	62	240 rPLU4
rFAL1	TTAAACTGGTTTGGGAAAACCAATATATT ACACAATGAACCAATCATGACTACCCGCT	<i>Plasmodium falciparum</i>	58	205 rFAL2
rMAL1	ATAACATTAGTTGTACGTTAAGAATAACCGC AAAATTCCCATGCATAAAAAATTATACAA	<i>Plasmodium malariae</i>	58	144 rMAL2
rOVA3*	CGGGGAAATTTCTTAGATTGC GAGAAACAGCATGAATTGCG	<i>Plasmodium ovale</i>	58	456 rOVA4

Plasmodium vivax was not tested because it is absent in Senegal where no study has shown its presence.

Calculation of the RDT parameters

PCR was used as a gold standard. Sensitivity, specificity as well as the predictive positive and negative values (PPV and NPV) were calculated. Sensitivity was equal to $TP/(TP+FN) \times 100\%$ and specificity was equal to $TN/(TN+FP) \times 100\%$. The PPV was obtained using the formula $TP/(TP+FP) \times 100\%$ whereas the NPV was obtained from $TN/(TN+FN) \times 100\%$. TP was the number of true positives (blood smear and RDT positives), TN the number of true negatives (blood smear and RDT negatives), FP the number of false positives (blood smear negative and RDT positive) and FN the number of false negatives (blood smear positive and RDT negative).

Results

At the end of the study, 273 patients had been involved. The average age was 11.5 years [1–70 years] with a sex-ratio of 1.03. The proportion of patients with prior antimalarial treatment was 28.4%. The average time before resorting to medical care was 2.5 ± 1.5 days. Variations ranging between 1 and 7 days were noted. The reading and interpretation of the RDT was done for all patients. In total, the result of the RDT was positive for 234 patients (85.7%). No invalid result was noted.

Microscopy on the field allowed for the confirmation of 223 (81.6%) positive thick smears (Table 2). Parasite density varied between 120 and 231 238 trophozoites per microliter of blood, with an average 10 540 trophozoites per microliter of blood. In the 222 patients, parasite infestation was with a single species. Only *P. falciparum* was found. One patient showed a combined infection with *P. falciparum* associated *P. malariae* (0.4%). *Plasmodium ovale* was not found.

Pan-malarial PCR (*Plasmodium* sp) was performed on the 273 RDTs of which 87.9% (240/273) were positive. Specific PCR confirmed *P. falciparum* in 99.6% (239/240) of the positive samples (Fig. 1A). *Plasmodium malariae* was not found (Fig. 1B). *Plasmodium ovale* was found in one patient (Fig. 1C) in a co infection with *P. falciparum*. In one case (0.4%), species could not be determined.

Table 2 Comparison between RDT *Malaria Antigen P.f* results versus Thick smear and PCR

RDT	Thick smear			PCR		
	Positive	Negative	Total	Positive	Negative	Total
Positive	219	15	234	233	1	234
Negative	4	35	39	7	32	39
Total	223	50	273	240	33	273

Calculation of the RDT parameters

The analysis involved 273 samples for which PCR results were collected. Discrepancies were found among these results (Table 2). A comparison between the RDT and the thick smear showed 19 discrepancies (4 false negatives and 15 false positives). No history of anti malaria treatment was recorded among these patients. PCR on the RDT strips showed 8 discrepancies (1 false positive and 7 false negative) compared to the results of the RDT. A likely prior anti malaria treatment was noted in the false positive patient. Discrepancies are presented in Table 3. The sensitivity of the RDT *Malaria Antigen P.f*® in relation to the PCR was 97.1% with a 96.9% specificity. VPP and VPN were 99.5% and 82% respectively. Compared to the thick smear, the sensitivity of the RDT was 98.2% (Table 4).

Discussion

Since several years, RDT has become an useful tool for malaria diagnosis especially in poor countries where malaria is endemic with a lack of well equipped laboratories. In Senegal, RDT was introduced since 2008 as a strategy in the management of malaria cases. The last report from the NMCP showed a drastic reduction of malaria cases¹ since this date and a reduction of anti malarial consumption was observed showing the cost effectiveness of this strategy.⁸ Becoming an indispensable tool for malaria diagnosis, RDT needs to be closely monitored to ensure good reliability. This assessment highlighted the good performance of the *Malaria Antigen P.f*® RDT. Indeed, we compared it to the thick smear and then we performed a PCR on the nitrocellulose strip it contains. Only the side of the strip where the blood is laid during the diagnosis test was used to that effect. These used RDTs were stored at room temperature, in no special conditions. The DNA that was extracted after a year using Chelex 100® (Bio-Rad Laboratories, Inc, Hercules, CA, USA) showed good sensitivity of this PCR to *Plasmodium* sp. Indeed, this PCR is highly sensitive even when parasitemia is low^{9,10} and it allowed us to detect more positives than the thick smear did. A good level of sensitivity was also achieved with the specific PCR.

This also confirms the stability of the parasite DNA on the nitrocellulose band, which makes it useful for PCR-based studies as demonstrated in earlier research.^{3,4,11} Carrying out a PCR from that strip contained in the test kit could therefore be a way to perform quality control on the results of the RDT. Indeed, even where the test has not detected the parasite either because it has been deteriorated (inadequate storage before it was used) or because it has been used incorrectly, the DNA present on the strip can still be extracted. In the same way, in case of

Figure 1 profiles obtained after specific PCR.

Table 3 Discrepancies between RDTs versus microscopy and RDT versus PCR

RDT negative	Thick smear	PCR
Patients with RDT negative and thick smear positive	Parasitemia	Plasmodium species
Patient 1	188 p/μl	P. falciparum
Patient 2	342 p/μl	P. falciparum
Patient 3	152 p/μl	P. falciparum
Patient 4	226 p/μl	P. falciparum
Patients with RDT negative and PCR positive	Parasitemia	Plasmodium species
Patient 1	Negative	Not determined
Patient 2	174 p/μl	P. falciparum
Patient 3	126 p/μl	P. falciparum
Patient 4	202 p/μl	P. falciparum
Patient 5	134 p/μl	P. falciparum
Patient 6	Negative	P. falciparum
Patient 7	Negative	P. falciparum

Table 4 Characteristics of Malaria Antigen P.f[®] RDT versus Thick smear and PCR

Test	Sensitivity	Specificity	PPV	NPV
RDT/PCR	97.1%	96.9%	99.5%	82%
RDT/Thick film	98.2%	70%	93.5%	89.7%

false positivity due to the persistence of the parasite antigen in the blood, confirmation can be obtained through a PCR. The *Malaria Antigen P.f[®]* RDT can only pick up *P. falciparum*. Infection by another species therefore entails a negative result.

Specific PCR has been done on these samples and the results were as good as those obtained from a PCR performed on regular blood or on filter papers.^{12,13} *Plasmodium ovale* has been identified by PCR. The RDT was positive for the patient hosting this species because of a co infection with *P. falciparum*. However, the RDT returned a negative result for the patient where the species has not been determined. In the countries where *P. falciparum* is predominant, such as Senegal, a RDT which detects HRP2 remains very relevant. However, difficulties in diagnosing species by microscopy can be common due to their rarity.

Compared to the thick smear and the PCR, the *Malaria Antigen P.f[®]* RDT showed good sensitivity with 98.2% and 97.1% respective rates which confirm its robustness.^{14,15} Recent quality control studies on RDTs also demonstrated a good level of sensitivity to low parasitemia.^{16–18}

Compared to PCR, the level of sensitivity picked up was also a good 97.1%. A false positive was detected, showing a level of specificity at 96.9% against 70% when compared to a thick smear. Better results were noted in other research.¹⁹

PCR also allowed the detection of 7 false negatives, 4 of which were also detected by microscopy. This could be explained by low parasitemia which is frequent in low transmission areas such as Senegal or by the genetic diversity of gene HRP2 in these parasite strains demonstrated by other research.^{20–22} This indicates the need for further research to measure the prevalence of deletion in this gene in the *P. falciparum* strains that can be found in the country. Research in Mali, a neighboring country to Senegal showed a 45% deletion prevalence in gene HRP2 in false negative patients with the ParaSight RDT.²³

It is however necessary to point out that despite a good capacity to extract DNA, the method using Chelex has restrictions as to the quality of the resulting DNA. This could have an impact on the results of the PCR.

In our research, variations in parasitemia ranged between 120 trophozoites/μl of blood as a minimum

and 231 238 as a maximum. The *Malaria Antigen Pf* RDT has therefore been able to detect parasitemia below 200 parasites/μl.

All along the study, the *Malaria Antigen Pf[®]* was kept at room temperature, in compliance with the manufacturers recommendations (manufacturer's standards allow temperature variations between 1 and 40°C). This mode of storage has not affected the operation of the RDT. This ease of storage is a useful feature of *Malaria Antigen Pf* RDT, especially for poorly equipped health units. However a quality control system must be in place to monitor the performance and quality of the RDT overtime in real life conditions of storage and use.

This evaluation shows that the *Malaria Antigen Pf[®]* RDT can be a useful tool for the detection of new clinical cases of malaria. Beyond the diagnosis aspect, the PCR on the strip is an excellent tool for quality control on RDTs. It is also useful for molecular research such as the monitoring of molecular markers of resistance to antimalarial drugs. Indeed, lower malaria related morbidity in many countries makes it more and more difficult to collect sufficient quantities of parasites for molecular research. Rapid diagnosis tests used in routine activities by health structures can help solve this problem.

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