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RAPPORT GLOBAL

EVALUATION EXTERNE DE LA QUALITE
DES ANALYSES EN BIOLOGIE CLINIQUE

COMMENTAIRE CONCERNANT L'ENQUETE TESTS RAPIDES MALARIA

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Evaluation externe de la qualité portant sur l'utilisation des tests de diagnostic rapide du paludisme dans une région non endémique

Résumé

Contexte

Les Tests Diagnostique Rapide (TDR) du paludisme sont de plus en plus utilisés tant en régions endémiques qu'en régions non endémiques. Ce rapport présente les résultats d'une session d'Evaluation Externe de la Qualité (EEQ) des TDR du paludisme dans une région non endémique.

Méthodologie

Après validation de la stabilité des antigènes lors d'un envoi à température ambiante, trois échantillons biologiques ainsi qu'un questionnaire ont été expédiés aux laboratoires d'analyses de biologie médicale de Belgique et du Grand Duché de Luxembourg. Il était demandé aux participants d'une part de rapporter les résultats du TDR en en termes d'intensité des bandes, tant pour le contrôle que pour le test, et d'autre part de rapporter les résultats du TDR tel qu'il est d'habitude envoyé au clinicien. De plus, les participants étaient invités à répondre à un questionnaire portant sur la place des TDR dans leur stratégie du diagnostic du paludisme.

Résultats

Au total, 128 des 133 (96.2%) laboratoires d'analyses biomédicales utilisant des TDR ont participés à cette évaluation. Six marques de TDR à 3 bandes et une à 4 bandes étaient utilisées. Les erreurs analytiques étaient rares et comprenaient (i) la non reconnaissance d'un résultat invalide en absence de la bande de contrôle (1.6%) et (ii) la non reconnaissance de *Plasmodium falciparum* (0.8%). Les erreurs mineures étaient liées à l'interprétation du résultat du TDR et comprenaient (i) le rapportage "TDR positif" sans mention de l'espèce *P. falciparum* ou *Plasmodium* non-*falciparum* (16.9% et 6.5% respectivement) et (ii) l'ajout de commentaires inappropriés au rapport (3.2%). Certaines de ces erreurs étaient associées aux instructions incorrectes données par le manuels d'utilisation du TDR tels que (i) l'omission d'inclure la possibilité d'une infection mixte dans le cas de *P. falciparum* et *P. vivax* (35.5% et 18.5% respectivement) et (ii) l'interprétation d'une infection à *Plasmodium vivax* à la place d'une infection à *Plasmodium* non-*falciparum* en présence d'une bande pan-spécifique (4.0%). Selon le questionnaire, 48.8% des participants ont traités moins de 20 demandes d'analyses paludisme en 2009. Si pendant les heures de travail, 95.2% des 125 participants utilisent les TDR en association à la microscopie, en dehors des heures de travail, 31.0% de 113 participants se basent uniquement sur les TDR pour le diagnostic du paludisme avec une microscopie reportée au lendemain ou non réalisée.

Conclusion

En région non endémique, les erreurs dans l'utilisation des TDR du paludisme sont principalement des erreurs d'interprétation de résultats. Certaines de ces erreurs d'interprétation proviennent d'instructions incorrectes décrites dans les manuels d'utilisation des TDR. L'usage des TDR comme méthode de diagnostic primaire ou unique du paludisme devrait être évité.

Table 1: Aperçu des TDR malaria en usage en Belgique (2010)

Fabricant	**Dénomination**	**Format**	**Antigènes ciblés**	**Nombre de laboratoires utilisant ce kit (%)**
Inverness Medical Binax, Inc., Sacrborough, Maine, USA	BinaxNOW® Malaria Test	Carton/dipstick Trois-bandes	HRP-2 + Aldolase	54 (42.1)
All Diag, Strasbourg, France	Palutop+4®	Cassette Quatre bandes	HRP-2 + Pv-pLDH + pan-pLDH	26 (20.3)
DiaMed AG, Cressiers/Morat Switzerland	OptiMal-IT or OptiMal	Hybride cassette/dipstick Trois-bandes	Pf-pLDH + pan-pLDH	23 (18.0)
Access Bio Inc, New Jersey, USA	CareStart™ Malaria pLDH/HRP2 Combo test	Cassette Trois-bandes	HRP-2 + pan-pLDH	12 (9.4)
Standard Diagnostics Inc, Hagal-Dong, Korea	SD Bioline Malaria Ag Pf/Pan FK 60	Cassette Trois-bandes	HRP-2 + pan-pLDH	11 (8.6)
Ultimed Ahrensburg, Germany	Malaria (*P. falciparum* / pan*)* Test	Cassette Trois-bandes	HRP-2 + pan-pLDH	1 (0.8)
Cypress Diagnostics, Leuven, Belgium	Malaria Total Quick Test	Cassette Trois-bandes	HRP-2 + pan-pLDH	1 (0.8)

Tableau 2: Quelles contributions les Tests de Diagnostic Rapides (TDRs) peuvent-ils avoir dans le diagnostic du paludisme?

Exigences pour le diagnostic du paludisme	**Contribution des TDRs**	**Commentaires**
Confirmation dans le délai ou exclusion du diagnostic du paludisme avec référence en cas de doute.	Utilité considérable dans le diagnostic du paludisme.	Excellente sensibilité, particulièrement pour *P. falciparum* > 100 parasites/µl. Faux négatif pour *P. falciparum* à faible densité parasitaire (<100/µl), et plus rarement au dessus de 100/µl.
	N'exclut pas indéniablement le paludisme (microscopie aussi indispensable).	Certaines mutations HPR-2 peuvent ne pas être détectées. Effet prozone rare mais possible particulièrement pour les TDRs basés sur l'HRP-2. Aide modérée pour le diagnostic de *P. vivax* et aide limitée pour le diagnostic de *P. ovale* et *P. malariae* est due à un manque de sensibilité.
Diagnostic différentiel entre *P. falciparum* (Danger pour la vie) et les espèces non-*falciparum.*	Aide considérable pour l'identification of *P. falciparum.*	Infections mixtes rares mais pas à exclure si réactions positives conjointes des bandes d'antigènes de *P. falciparum*- et pan-species.
Evaluation de la densité parasitaire, reconnaissance particulière des valeurs critiques (>2% des globules rouges infectés).	Aucune aide.	L'intensité des bandes est indicative de la densité parasitaire mais pas toujours. Pour les tests P.f /pan, la présence unique de la bande HRP-2 peut indiquer une faible densité parasitaire.
Reconnaissance des stades de développement et de la présence d'hémozoïne pour *P. falciparum.*	Aucune aide.	

Tableau 3: TDR malaria, ce qu'il faut faire et ce qu'il ne faut pas faire.

Ce qu'il faut faire et ce qu'il ne faut pas faire dans l'utilisation des TDRs	**Commentaires**
Vérifiez la bande de contrôle – répéter le TDR si la bande de contrôle n'est pas visible.	L'absence de la bande de contrôle signifie un test invalide et aucune conclusion ne peut en être tirée.
Ne stockez pas les TDRs dans un congélateur.	La congélation détruit l'or-colloïdal.
Ne lisez pas le résultat avant ou après le temps de lecture recommandé par le fabricant.	Une lecture précoce peut donner un résultat faussement négatif. Une lecture tardive peut donner un résultat faussement positif.
Respectez le volume de sang recommandé par le fabricant (vous pouvez utiliser une pipette de précision au lieu du dispositif de transfert fourni dans le kit).	Trop peu de sang peut donner un résultat faussement négatif. Trop de sang entraine une diminution de l'éclaircissement de la membrane, et entrave la lecture (principalement pour des bandes de faibles intensités). Trop de sang peut augmenter le risque (et/ou l'intensité) de l'effet prozone.
Utilisez une pipette de précision.	Les dispositifs de transfert fournis dans les kits sont souvent petits et difficiles à manipuler.
Considérez une bande de faible intensité comme bande positive.	Toute bande visible est une bande positive.
Répétez un TDR négatif en cas de suspicion du paludisme.	Répétez le TDR après 8 à 12 heures jusqu'à 4 fois consécutive endéans les 36 heures pour exclure le paludisme.
N'utilisez pas les TDR pour suivre l'efficacité du traitement.	Les TDRs basés sur HRP-2 restent positifs pendant des semaines après une infection à *P. falciparum*. Les gamétocytes expriment la pLDH et l'aldolase.
Associez toujours le TDR à la microscopie	cf. Tableau 1.
Envoyez tout échantillon positif ou douteux au laboratoire de référence pour la confirmation (service gratuit).	Consultez le formulaire de demande et les instructions sur : https://www.iph.fgov.be/epidemio/epinl/plabnl/N_Plasmodium.pdf https://www.iph.fgov.be/epidemio/epifr/plabfr/F_Plasmodium.pdf

Comments to the results of the EQA SESSION 2009/3 on Malaria Rapid Diagnostic Tests

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In this comment, we briefly describe the backgrounds and methods of Malaria Rapid Diagnostic Tests (further abbreviated as MRDTs). Next, we will consider the present results of the External Quality Assessment (EQA) session in terms of analytical performance (the test results) and relevant reporting. We will address the strengths and weaknesses of MRDTs in the scope of travel medicine, and discuss appropriate diagnostic strategies.

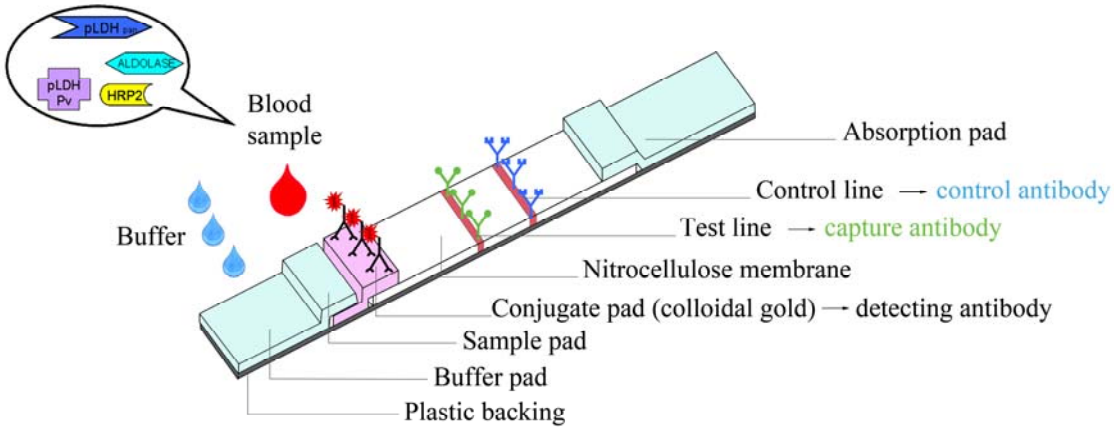
I. Background:

I.1. What are Malaria Rapid Diagnostic Tests? How do they work?

MRDTs detect *Plasmodium* parasites in blood by an antibody-antigen reaction on a nitrocellulose strip. This strip can be available as a self-standing strip or be embedded in a plastic cassette or occasionally in a cardboard format. Reactions on the nitrocellulose strip are visible as cherry-red lines. Two-band MRDTs are mostly designed to detect *Plasmodium falciparum*; they display a control line and a test line which targets either histidine-rich protein-2 (HRP-2) or *P. falciparum*-specific parasite lactate dehydrogenase (Pf-pLDH). Three- and four-band MRDTs display a control line and two or three test lines, one targeting a *P. falciparum* specific antigen, another line targeting antigens common to the four species such as pan-*Plasmodium*-specific lactate parasite dehydrogenase (pan-pLDH) or aldolase, and, in case of the four band MRDTs, a third line which targets *Plasmodium vivax*-specific pLDH (Pv-pLDH).

HRP-2 is a protein produced by asexual stages and young gametocytes of *P. falciparum*. It is expressed on the red blood cell membrane surface and readily diffuses into the plasma. pLDH is an enzyme in the glycolytic pathway of the *Plasmodium* spp., and is produced by sexual and asexual stages of the parasite. Aldolase is another enzyme of the *Plasmodium* glycolytic pathway that is also used as a target for detection. Of note is that the HRP-2 antigen can persist for up to several weeks in the blood after successful treatment (due to a low clearance from the blood), whereas pLDH and aldolase depend on living parasites and disappear from the circulation upon treatment (Moody, 2002).

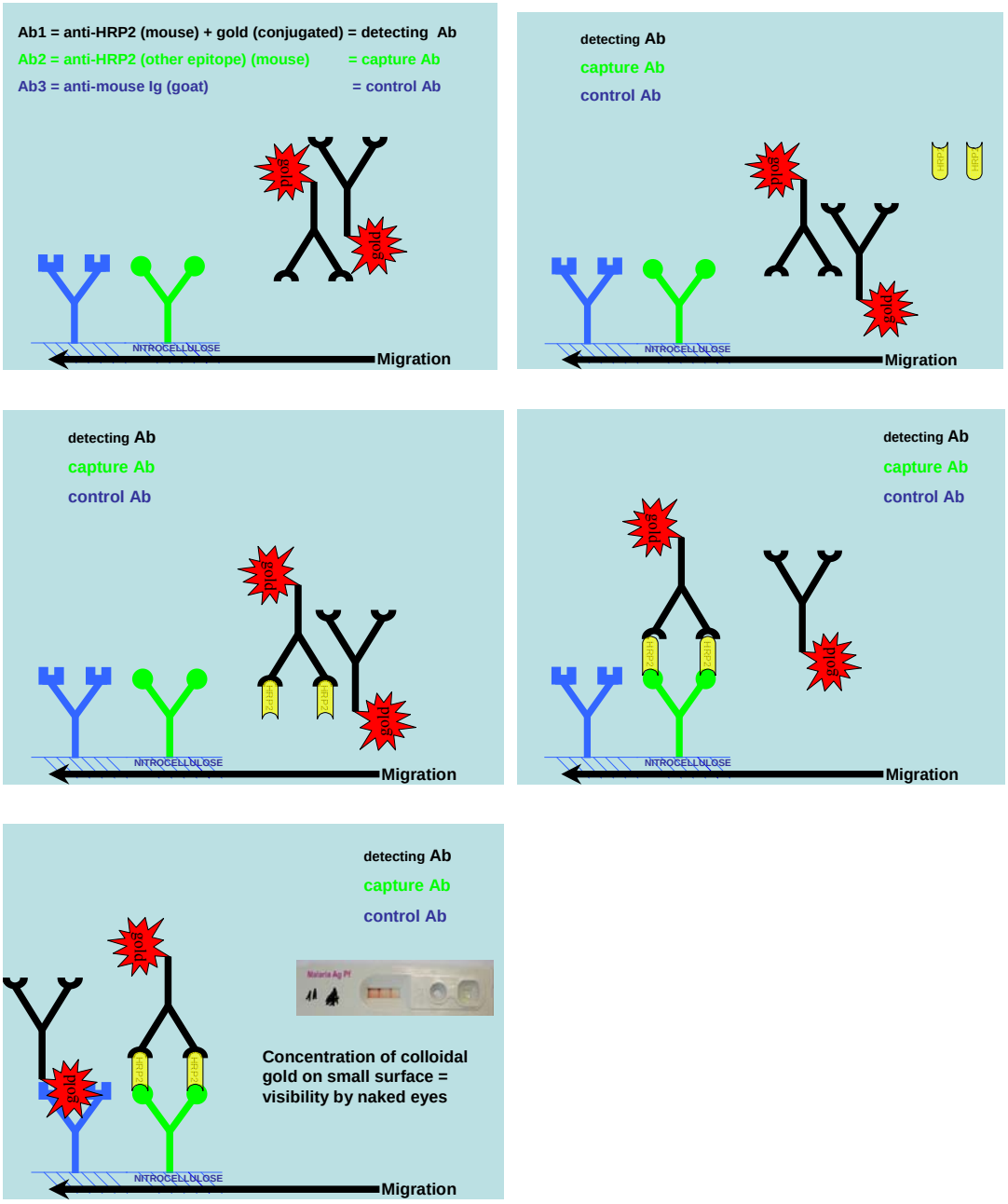
Figure 1: Schematic drawing of the MRDT lateral-flow strip.



Figures 1 and 2 show the different components of a MRDT strip and the sequence of reactions on a two-band MRDT strip respectively. The patient's blood and several drops of buffer are applied respectively to the sample and buffer pad of the strip. They are attracted by the capillary action of an absorption pad at the other end of the strip and start to migrate. First, they pass the so-called conjugate pad, which contains a detection antibody targeting a *Plasmodium* antigen, such as HRP-2, Pf-pLDH, Pv-pLDH, pan-pLDH or aldolase. This detection antibody is a mouse-antibody that is conjugated to a signal, mostly colloidal gold. If present in the sample, the *Plasmodium* antigen is bound to this detection antibody-conjugate. Next, the antigen-antibody-conjugate complex migrates further across the strip until it is bound to a second antibody, the so-called capture antibody. This capture antibody binds to another epitope of the *Plasmodium* target antigen. As the

capture antibody is applied on a narrow section of the strip, the complex with the conjugated signal will be concentrated and by virtue of the colloidal gold will become visible as a cherry-red colored line. The excess of detection antibody-conjugate that was not bound by the antigen and the capture antibody moves further towards the absorption pad until it is bound to a goat-raised anti-mouse antibody, thereby generating a control line (Bell *et al.*, 2006; Moody, 2002).

Figure 2: Two-band MRDT targeting HRP-2: consecutive reaction steps**.**



MRDTs use a simple but robust technique which has many other applications (HIV screening, pregnancy test, screening tests for drug use, rapid diagnostic tests for influenza, RSV etc). The technique as described above is frequently referred to as "immunochromatography", and MRDTs are also referred to as "lateral flow tests" as opposed to the "flow-through tests". In the former tests, the sample moves along the nitrocellulose strip whereas in the latter it moves through the membrane. An example of such a test system is the HIV spot**,** Genelabs Diagnostics, Singapore (one of the first rapid tests used for the detection of HIV antibodies, Figure 3). Lateral flow tests should neither be named "dipstick tests", because in dipstick tests there is no flow of the sample along the strip: the reaction is read at the place of the sample application. Due to their simple design and intrinsic robustness, MRDTs are heat-stable (until 30°C or even 40°C for some of them) and do not need a cold chain (in contrast to, for instance, latex agglutination tests). In addition, the technique allows for a long shelf-life (mostly more than 18 months). The colloidal gold conjugate however will be damaged at temperatures below 0°C.

Figure 3: HIV SPOT test, example of a "flow-through test**".**

In malaria-endemic regions, MRDTs are currently rolled out by National Malaria Control Programs as a tool for parasite based diagnosis (Drakeley & Reyburn, 2009). As they are simple to perform, they can also be carried out by non-specialized health care workers (Mayxay *et al.*, 2004; Rennie *et al.*, 2007). In non-endemic settings, where microscopic expertise is lacking due to low incidence, they are used as an adjunct to microscopy, especially outside opening hours but also as bedside point of care tests (Chilton *et al.*, 2006; Wiese *et al.*, 2006), and they have been reported to perform accurately and even better as compared to microscopy (Palmer *et al.*, 2003; Stauffer *et al.*, 2009). In addition, MRDTs are marketed for self-use by travelers (Risch *et al.*, 2000). In 2007, more than 70.000.000 tests were performed (Bell & Perkins, 2008) and more than 80 brands are marketed world-wide (Drakeley & Reyburn, 2009).

I.2.What were the objectives of the present External Quality Assessment session?

The World Health Organization (WHO) has set up a comprehensive quality control strategy for the MRDT products (such as quality control of production, transport and product control at the level of National Reference Laboratories (WHO, 2009b; WHO, 2010c), but there is few guidance for quality assurance at the level of the end user (de Oliveira *et al.*, 2009; McMorrow *et al.*, 2008). Although MRDTs are currently widely used in non-endemic settings, no formal EQA sessions on the use of MRDTs have been organized. However, a national survey in the U.K. highlighted the need for such EQA sessions from the part of clinical laboratories (Chilton *et al.*, 2006). In light of these reasons, the present EQA was organized.

II. Methods

II.1. Participants, samples and questionnaire

An inquiry of the Institute of Public Health (IPH) revealed that 128/183 (69.9%) of participants subscribing to the EQA "Microbiology" in 2009-2010 were also interested in an EQA on MRDTs. In a survey afterwards, the non-subscribing laboratories were addressed to ask whether they use MRDTs or not in the diagnosis of malaria. When surveyed, 50 of the 55 non-subscribing laboratories declared not to perform RDTs as part of malaria diagnosis. In summary, 133/183 (72.7%) of clinical laboratories offering malaria diagnosis were using RDTs at the time of EQA, and 128 (96.2%) of them participated to the present EQA session on malaria RDTs.

Three clinical samples were selected. They were obtained from patients suspected of malaria presenting at the Institute of Tropical Medicine (ITM).

Diagnosis of malaria, species identification and determination of parasite density were done by microscopy. According to standard practice at the ITM, thick and thin blood films were prepared, stained with Giemsa (pH 8.0) and examined by light microscopy using a × 500 magnification (Van der Palen *et al.*, 2009). Parasite densities were estimated by counting asexual parasites against 200 white blood cells (WBC) in thick blood films, converting this number to parasites/µl using the actual WBC count (Moody, 2002). Parasite densities are further in this text expressed as counts (of asexual parasites)/µl (of whole blood), with 50,000 red blood cells/µl set as 1% of red blood cells (Moody, 2002). Species identification was confirmed by *Plasmodium*-specific PCR (Cnops *et al.*, 2010). Laboratory diagnosis of malaria at ITM is accredited in accordance with the requirements of the standard NBN EN ISO 15189:2007.

The EQA panel consisted of three samples: one sample with *P. falciparum*, another with no evidence of *Plasmodium* and a third one with *P. vivax*. Table 1 displays the clinical information and parasite densities of the samples. All three samples were assessed at ITM at the time of diagnosis with the panel of MRDTs listed in Table 4, except for the Cypress and Ultimed brands, which were tested on stored samples at the time of EQA shipment validation.
After initial analysis and diagnosis, samples were stored at 4°C for a maximum of 48 hours and subsequently aliquoted in 150 µl-fractions which were stored at -70°C. Total durations of storage at -70°C till EQA shipment were 612, 249 and 240 days for samples 10085, 10086 and 10087 respectively.

Table 1: Clinical information and parasite density of the embedded samples of the EQA session.

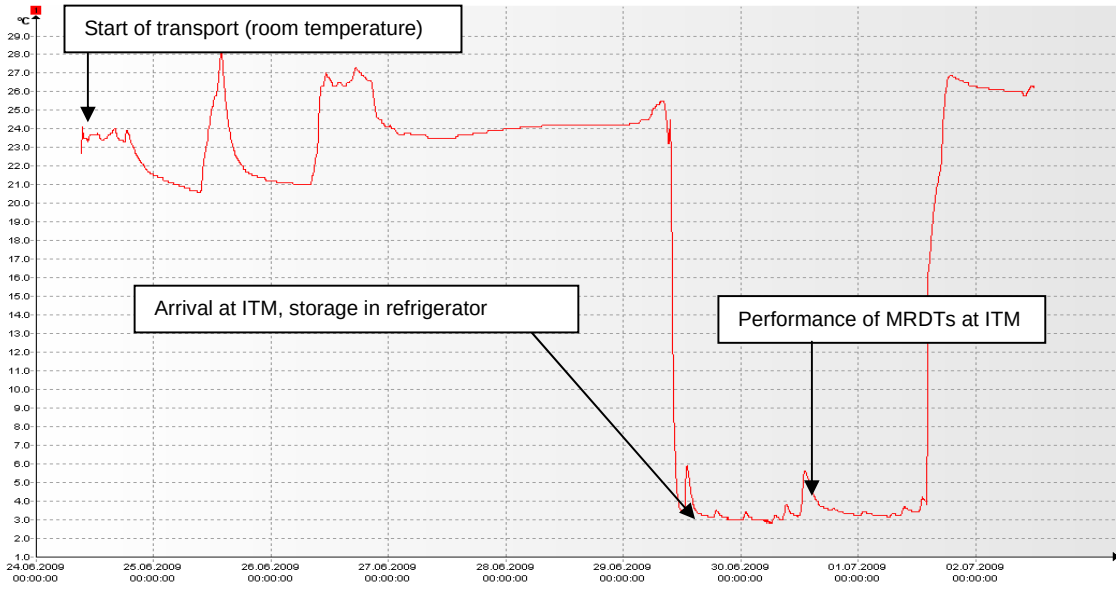
Sample number	History	Species, parasite density
10085	Pregnant woman, Nigeria.	*P. falciparum*, 53,024/µl = 1%
10086	NGO volunteer, Burkina Faso, treated for malaria 4 weeks ago.	No *Plasmodium* detected.
10087	Traveler: Democratic Republic of the Congo, Haiti.	*P. vivax*, 3,251/µl = 0.06%

Participants were asked to state the MRDT brand name they used and to report their results in terms of observations and interpretations. Observations included the raw results, *i.e.* the presence of visible control and test lines with a score for test line intensities (strong, medium, weak and faint in comparison to the control line) (Van der Palen *et al.*, 2009). Interpretations referred to the final diagnosis: participants were offered a free-text option and invited to submit their reports to the clinician as they would do in daily practice. In addition to the samples, a questionnaire on the indications and performance on MRDTs was prepared. This questionnaire was based on a previous survey performed in the United Kingdom and addressed issues of diagnostic strategy (Chilton *et al.*, 2006). For analysis, the results of the MRDT tests and the answers to the questionnaire were reviewed and grouped.

II.2. Validation of shipment and questionnaire

For validation of the samples (in particular their antigen stability), questionnaire and instructions, a try-out EQA session was organized among the members of the IPH referee committee, which consisted of a panel of 9 laboratory professionals in charge of pilot evaluation of EQA sessions on parasitology. Aliquots of the samples were retrieved, allowed to thaw and processed at room temperature. One aliquot was tested again against the panel of MRDTs. The other aliquots were packaged according to the UN 3373 recommendations and transported the next day by car to a regular IPH referee committee meeting. Each member received two packages: one was prepared for analysis by his/her laboratory; the other was prepared to be sent back by regular post to ITM. The latter package contained a temperature logger (Escort data loggers®, Buchanam, Virginia United States) allowing to monitor temperature during transport and shipment (Figure 4).

Figure 4: Temperature monitoring during validation of the EQA shipments



In addition, each referee received a copy of the questionnaire which had to be filled in and returned to ITM.

Upon arrival at ITM, the packages with the samples were stored at 4°C for a maximum of 48 hours and MRDT testing was repeated. Test line results obtained by MRDT testing of aliquots before shipment and after return were compared and proved to be identical. All MRDTs were performed according to the instructions of the manufacturer, except that the transfer straws or loops supplied in the MRDTs' kit were replaced by a transfer pipette (Finnpipette, Helsinki, Finland). Readings were carried out at daylight assisted by a standard electric bulb by three subsequent observers, recording test line intensities (Van der Palen *et al.*, 2009). All MRDT kits had been stored between 18°C and 24°C before use.

II.3. Interpretation of results and data analysis

Participants entered the results online via the IPH-EQA website or sent their results by ordinary mail to the IPH. These results were converted or encoded in an excel database. The results of the MRDTs and the answers to the questionnaire were reviewed and grouped. As we focused on the number of participants as the denominator, we considered the first MRDT in case a participant used more than one brand. We primarily considered the final test report (the report as assumed to be sent to the attending clinician). In case of unexpected results, we retrieved the observations (visibility and intensity of test and control lines) in order to distinguish between interpretative versus analytic errors.

Interpretation and scoring of the MRDT results was done based on what we considered essential in routine diagnosis pending referral to the reference laboratory, as outlined in Table 2.

Table 2: Laboratory diagnosis of malaria: expected minimal performance in the reference setting and in routine diagnosis.

Reference laboratory	Daily patient care, routine diagnosis
Timely confirmation or exclusion of the diagnosis of malaria.	Timely confirmation or exclusion of the diagnosis of malaria, with prompt referral in case of doubt.
Species identification.	Distinction between *P. falciparum* and the non-*falciparum* species.
Assessment of parasite density, expressed as numbers of asexual parasites/µl.	Assessment of parasite densities, recognition of critical values (>2% of red blood cells infected).
Recognition of the *Plasmodium* stages and malaria pigment (hemozoin) in white blood cells.	

Differentiation of *P. falciparum* from the non-*falciparum* species, in particular not missing the diagnosis of *P. falciparum* is important because of the life-threatening potential of this species. Accurate identification of parasite density appears to be difficult in non-endemic settings (Kettelhut *et al.*, 2003), but high parasite

densities exceeding 2% of red blood cells infected should be recognized, as this criterion constitutes an alert sign (WHO, 2010a). Expert microscopy should also recognize *P. falciparum* stages and hemozoin pigment in WBC: schizonts of *P. falciparum* in the peripheral circulation as well as hemozoin in the WBC in case of *P. falciparum* infection are indicators of a serious infection, whereas the exclusive presence of *P. falciparum* gametocytes after treatment is a normal finding (Grobusch *et al.*, 2003; WHO, 2010a).

Based on these criteria, we ranked errors into three categories: very minor, minor and major errors (Table 3). The score for the diagnostic strategy of malaria is explained further in the text.

Table 3: Score for EQA test results, considered as “report to the clinician”.

Correct	• Correct diagnosis and correct report.
Very minor error	• Not diagnosing or reporting the possibility of a mixed infection, with non-*falciparum* species as the disregarded species.
Minor error	• Missing the diagnosis of non-*falciparum* species. • Reporting "positive" when information on confirmation/ruling out of *P. falciparum* is available. • Reporting *P. vivax* in stead of non-*falciparum* species. • Correct result but with incorrect comment.
Major error	• Invalid MRDT test result not recognized. • Diagnosis of *P. falciparum* missed. • *P. falciparum* diagnosed or reported as non-*falciparum* species. • Non-*falciparum* species diagnosed or reported as *P. falciparum.* • Negative sample diagnosed or reported as “positive”.

III. Results

III.1. Overview of the MRDT kits used by the participants

Table 4 lists the different MRDTs used by the participants, matched with their format and target antigens. The shortened names that are further used to refer to the different MRDT brands are underlined. We added the single "Palutop" name to the "Palutop+4" group (n = 25) as it was clear from the raw test results that it was the same MRDT brand. Likewise, we grouped together the two OptiMAL products: OptiMAL-IT (n = 21, with "IT" standing for "individual test" referring to the individually wrapped packages) and OptiMAL (referring to the kit package, n = 2) as these kits have identical components and perform equally well (Moody, 2002).

Table 4: Overview of malaria MRDTs used by the participants (n=128).

Manufacturer	Malaria MRDT	Format	Target antigens	Numbers (%)
Inverness Medical Binax, Inc., Sacrborough, Maine, USA	BinaxNOW® Malaria Test	Card box Three-band	HRP-2 + Aldolase	54 (42.1)
All Diag, Strasbourg, France	Palutop+4®	Cassette Four-band	HRP-2 + Pv-pLDH + pan-pLDH	26 (20.3)
DiaMed AG, Cressier s/Morat Switzerland	OptiMal-IT or OptiMal	Hybrid dipstick Three-band	Pf-pLDH + pan-pLDH	23 (18.0)
Access Bio Inc, New Jersey, USA	CareStart™ Malaria pLDH/HRP2 Combo test	Cassette Three-band	HRP-2 + pan-pLDH	12 (9.4)
Standard Diagnostics Inc, Hagal-Dong, Korea	SD Bioline Malaria Ag Pf/Pan FK 60	Cassette Three-band	HRP-2 + pan-pLDH	11 (8.6)
Ultimed Ahrensburg, Germany	Malaria (*P. falciparum* / pan) Test	Cassette Three-band	HRP-2 + pan-pLDH	1 (0.8)
Cypress Diagnostics, Leuven, Belgium	Malaria Total Quick Test	Cassette Three-band	HRP-2 + pan-pLDH	1 (0.8)

The underlined names represent the shortened names used in the text to refer to the different MRDT brands

For expression of results, we did not take into account the *P. vivax*-specific pLDH test (SD P. vivax FK 70, Standard Diagnostics) used by a single participant for sample 10087 nor the two MRDTs that were used respectively as a second MRDT by one participant and as a MRDT kit under evaluation by another participant. Both MRDTs (Palutop and SD Bioline respectively) gave the expected test results for all three samples

III.2 Results for Sample 10085

Sample 100085:
"Nigerian woman, 28 years old, pregnant for 24 weeks, did arrive from Nigeria two weeks ago. She is suspected of malaria, and did not take malaria prophylaxis"

Diagnosis: *P. falciparum*, parasite density 53,024/µl.

Expected MRDT Result:

- In case of a three-band test: "*P. falciparum*, a mixed infection with *P. vivax*, *P. ovale* or *P. malariae* can not be excluded".
- In case of the four-band Palutop: "*P. falciparum*, a mixed infection with *P. ovale* and *P. malariae* can not be excluded").

The results of the participants are displayed in Table 5.

Table 5: Results for sample 1: *P. falciparum* sample. Eligible answers of 124 participants were included.

	RDT brand						
Reported result	**Binax**	**Palutop**	**Optimal**	**CareStart**	**SD Bioline**	**Ultimed/ Cypress**	**Total (%)**
Negative*		1					1 (0.8)
Positive†	13	1	2	1	3	1	21 (16.9)
P. falciparum‡	1	22	10	7	4		44 (35.5)
P. falciparum or mixed infection	37	2	11	4	4		58 (46.8)

*major, † minor and ‡ very minor errors, see definitions in Table 3.

Comment:
Four participants did not give a final result in terms of interpretation and report to the clinician. Their observations of test lines were correct, but they were not included for analysis. The final denominator consisted of 124 participants.

Only a single major error was observed: one sample was reported as negative. The raw results (reported observations of test lines) for this particular sample, however, were correct (the presence of HRP-2 and pan-pLDH lines was reported); therefore we assume this was an administrative error.

Less than half (46.8%) of the participants scored correctly but another third (35.5%) committed the very minor error of disregarding the possibility of a mixed infection (non-*falciparum* species together with *P. falciparum*). In practice, this error has no impact on the short-term patient care: in case of an additional *P. vivax* or *P. ovale* species, the persistent liver schizonts have to be eradicated with primaquine treatment, but this information does not need to be known instantly and there is time to await confirmation from the reference laboratory. We however considered the simple "positive" report (16.9 %) as a minor error: indeed, all relevant information from the MRDT results - in this case the presence of the potentially fatal *P. falciparum* - should be exploited.

The unexpected high frequency (n = 44, 35.5%) of the very minor error of reporting *P. falciparum* without mentioning the possibility of a mixed infection with non-*falciparum* species was striking. Half of them were obtained with Palutop, representing the vast majority (22/26) of reports obtained with this brand. Looking at the package insert of this kit, it is clear that the error is embedded in the product instructions: Figure 5 shows that in case of visibility of both the "Pf" (HRP-2) and the "Pan" (pan-pLDH) lines, Palutop's instructions mention the diagnosis "*P. falciparum*", but not "*P. falciparum*, mixed infections with *P. ovale* and/or *P. malariae* not excluded". This is also the case for OptiMAL Rapid malaria Test (Figure 6). Other MRDTs such as OptiMAL IT (Figure 7) and SD Bioline (Figure 8) mention the possibility of a mixed infection, but the instructions are apparently not very clear or unequivocal. SD Bioline describes the "interpretation of test results" from the standpoint of the *Plasmodium* species: this approach generates a less clear overview compared to the systematic step-by-step interpretation from Binax based on the appearance of test lines (Figure 9).

Figure 5: Package insert of Palutop, test interpretation. The instructions do not mention the possibility of a mixed infection in case of "*P. falciparum* malaria" and "*P. vivax* malaria".

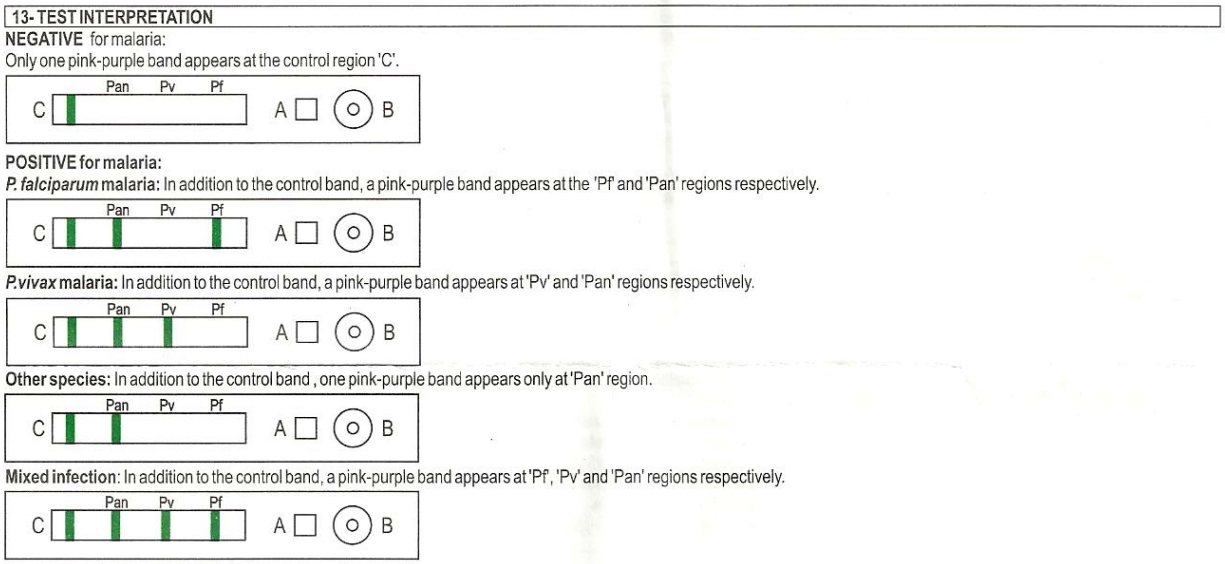


Figure 6: Package insert of OptiMAL, test line interpretation. The instructions do not mention the possibility of a mixed infection in case of "Positive for *P. falciparum*".

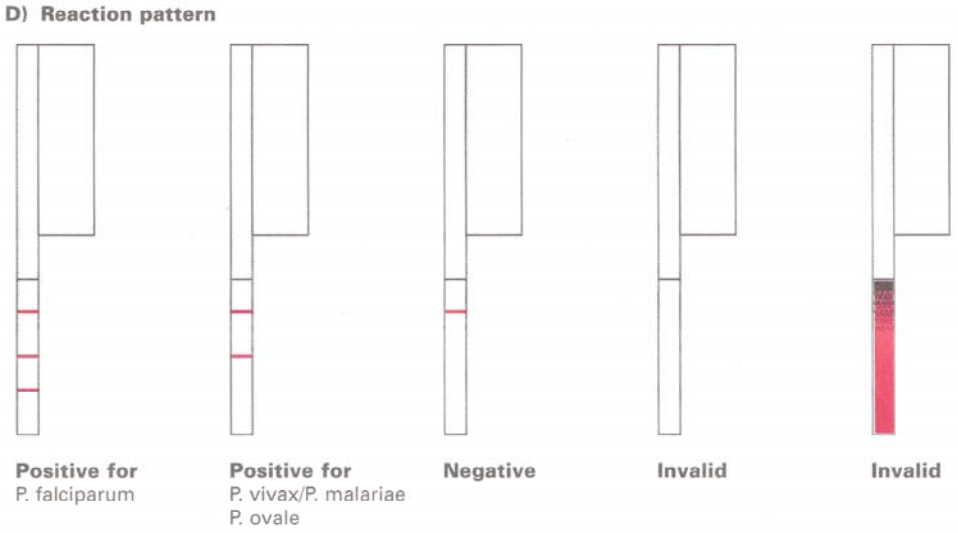


Figure 7: Package insert of OptiMAL-IT. The instructions do mention the possibility of a mixed infection in case of "Positive for *P. falciparum*".

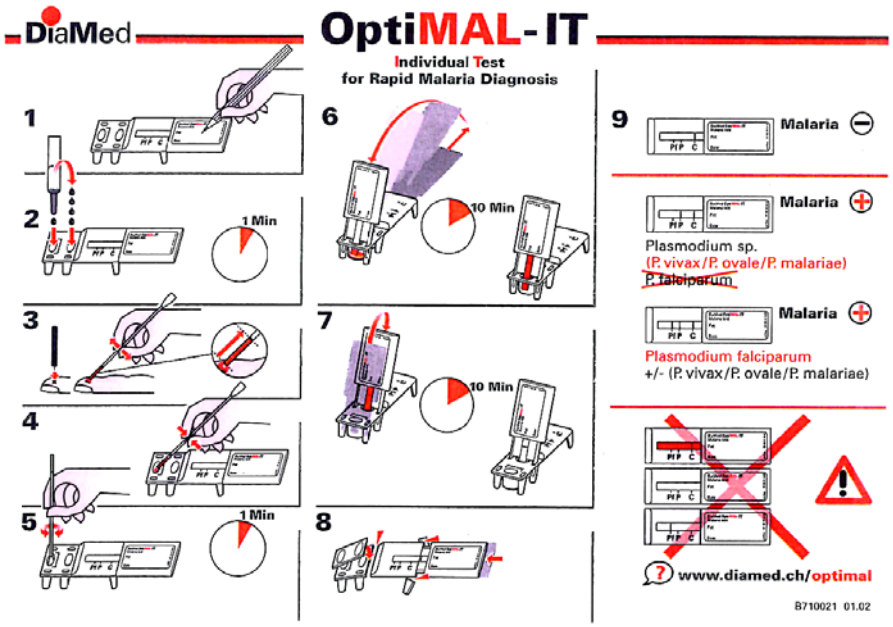


Figure 8: Package insert of SD Bioline, Test interpretations. Instructions are written from the standpoint of the *Plasmodium* species.

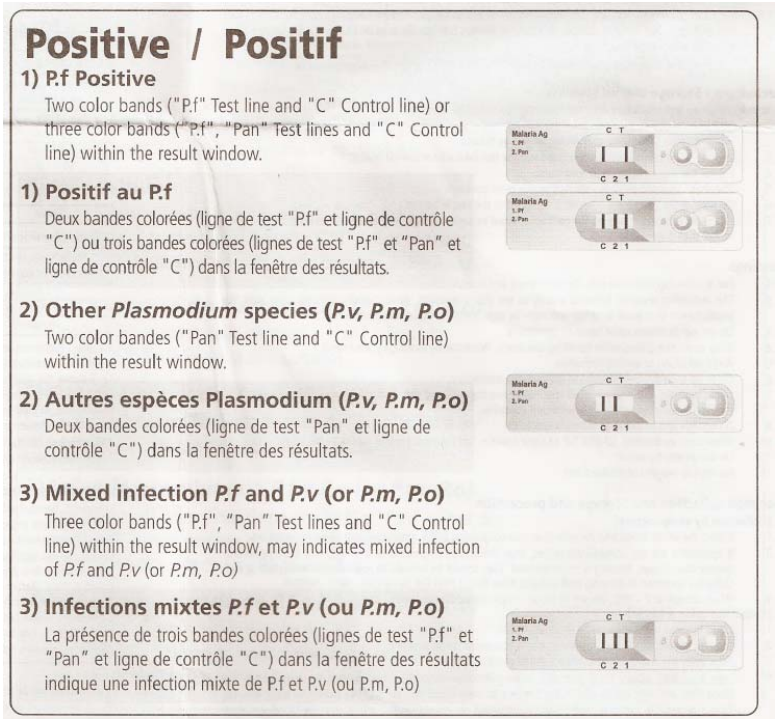
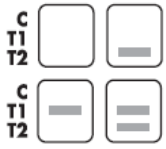


Figure 9: Package insert of Binax. Test line interpretations. Instructions starting from the observed test lines.

TEST	RESULTS	DESCRIPTION / INTERPRETATION
T1 Positive		Positive result for *P. falciparum* (P.f.)
T2 Positive		Positive result for *P. vivax* (P.v.) or *P. malariae* (P.m.) or *P. ovale* (P.o.) In some cases the appearance of only the T2 Line may indicate a mixed infection with two or more of P.v., P.m., and P.o.
T1 + T2 Positive		Positive result for *P. falciparum* (P.f.) In some cases the appearance of both the T1 and T2 Lines may indicate a mixed infection of P.f. with another species.
No T1 or T2 Lines		Negative result (no malaria antigens were detected)
Invalid and/or Uninterpretable Test Results		The test is invalid if the Control (C) Line does not appear, whether a Test Line(s) is present or not. The test is uninterpretable if the background color hinders reading of the test result at 15 minutes. Invalid or uninterpretable tests can occur due to improper sample or Reagent A addition. Consult the Test Procedure section and Precaution # 5 before repeating testing with a new device. Call Technical Service if the problem persists.

Although all but one MRDT generated the expected test lines, we would like to address some issues of sensitivity and related ones:

What is the detection limit of MRDTs for P. falciparum?
Expressed in parasite count per µl, most MRDTs have a detection limit of 100 asexual parasites (corresponding to 0.002% of red blood cells infected), and likewise comply with the WHO criteria of diagnostic sensitivity of at least 95% at this 100/µl threshold. This detection limit generally is above the threshold of expert microscopy (50/µl) but below that of routine microscopy in non-endemic settings, which has been shown to be close to 500/µl (Moody, 2002). Of note, non-immune travelers can present with symptoms at that parasite density (Murray *et al.*, 2008). Table 6 lists the parasite densities according to species for 1066 malaria-positive samples diagnosed at ITM during the period January 2000 – June 2010: for *P. falciparum*, 10% of samples at diagnosis had a parasite density below 100/µl. To give an idea about some of the currently used MRDTs evaluated in reference settings, we refer to the WHO and Foundation for Innovative New Diagnostics (FIND) evaluations of MRDTs (WHO, 2009a; WHO, 2010b) as well as studies performed by ITM (Maltha *et al.*, 2010; Van der Palen *et al.*, 2009; van Dijk *et al.*, 2009; WHO, 2009a; WHO, 2010b).

Table 6: Distribution of parasite densities (asexual parasites/µl) per species for the 1066 *Plasmodium* positive samples submitted to ITM for the period January 2000 - June 2010. (Only the first sample per patient was included).

Parasite density	**Single infection, species**				**Mixed infection**[†]
Numbers	***P. falciparum***	***P. vivax***	***P. ovale***	***P. malariae***	
0-100	81	3	12	3	0
101-500	93	14	22	3	2
501-5000	221	52	38	20	4
5001-250000	360	59	14	6	5
> 250000	53	1			
Total	808	129	86	32	11
Cumulative (%)					
≤ 100	10.0	2.3	14.0	9.4	0
> 100	90.0	97.7	86.0	90.6	100
> 500	78.5	86.8	60.5	81.3	81.8
> 5000	51.1	46.5	16.3	18.8	45.5
> 250000	6.6	0.01	0	0	0

[†] Mixed infections included *P. falciparum* infection with *P. ovale* (n=4) or *P. malariae* (n=5) and *P. malariae* infection with *P. ovale* (n=1) or *P. vivax* (n=1).

Can MRDTs generate information about parasite density?
In this session, we asked the participants to score test line intensities according to five categories: none, faint, weak, medium and strong (Van der Palen *et al.*, 2009)). In reference conditions, there are good inter-observer

correlations for reading categories of line intensities. In addition and not unexpectedly, test line intensities tend to be higher at increasing parasite densities (Gillet *et al.*, 2009a; Gillet *et al.*, 2009c; Maltha *et al.*, 2010; Van der Palen *et al.*, 2009; van Dijk *et al.*, 2009; van Dijk *et al.*, 2010). However, there is a big overlap between parasite densities for these line intensity categories, precluding their use as an indicator for parasite density – with the possible exception of a unique HRP-2 line (see below). Further, there may be differences in line intensities depending on the antigen targeted and on the particular MRDT brand.

In the present EQA, all but three (121/124, 97.6%) participants observed for sample 10085 both *P. falciparum*-specific (HRP-2 or Pf-pLDH line) and pan-specific *Plasmodium* lines (aldolase or pan-pLDH). In daily use of MRDTs, observations of single HRP-2 lines occur frequently: in some MRDT brands in case of *P. falciparum*, the antigen-antibody interaction for HRP-2 is stronger as compared to the interaction with the pan-specific antigen resulting in a lower detection limit for the HRP-2 and its single presence in case of low parasite densities (Murray *et al.*, 2008). Another consequence is that, generally speaking, the HRP-2 line will show stronger line intensities as compared to the pan-specific lines ((Van der Palen *et al.*, 2009; van Dijk *et al.*, 2009; van Dijk *et al.*, 2010). In the present EQA, this was apparent for Binax (49/54 strong line intensities recorded versus 9/54 for aldolase, (Mc Nemar change test, $p < 0.001$) and SD Bioline (11/12 strong line intensities for HRP-2 versus none for pan-pLDH, $p < 0.001$). Likewise, in *P. falciparum* samples with low parasite densities, only the HRP-2 band will be visible. Although there is some overlap, this situation may point to parasite densities below 1000/µl, such as in the case of SD Bioline, Palutop and CareStart (Maltha *et al.*, 2010; Van der Palen *et al.*, 2009; van Dijk *et al.*, 2009). In our experience, this phenomenon occurs only rarely with the Pf-pLDH in OptiMAL: among 257 *P. falciparum* samples tested positive with OptiMAL at ITM, we observed only three samples (1.2%) that reacted with the *P. falciparum*-specific Pf-pLDH and not with the pan-species pLDH. Of note, the presence of a single HRP-2 (not Pf-pLDH) line may also indicate a past *P. falciparum* infection, given the persistence of the HRP-2 antigen, as explained below.

A faint test line is a positive one

With regard to line intensities, it should be borne in mind that any line, irrespective of its intensity, represents a positive result. Indeed, misinterpretation of faint lines as negative results is a common mistake made by inexperienced staff, travelers and community health care workers, both in endemic and non-endemic setting (Harvey *et al.*, 2008; Mayxay *et al.*, 2004; Rennie *et al.*, 2007; Wiese *et al.*, 2006). MRDT brands that have been assessed for line intensities invariably show that HRP-2 lines display higher intensities as compared to Pv- and pan-pLDH specific lines and that the non-*falciparum* species (in particular *P. ovale* and *P. malariae*) generate lower line intensities as compared to *P. falciparum* ((Maltha *et al.*, 2010; Van der Palen *et al.*, 2009; van Dijk *et al.*, 2009; van Dijk *et al.*, 2010).

III.3. Results for Sample 10086

Sample 10086:
"(Expatriated) man, 25 years old is working as a volunteer in an agricultural project in Burkina Faso. He has received appropriate treatment for *P. falciparum* malaria 4 weeks earlier. He presents for a check-up.

Diagnosis: No *Plasmodium* antigen present (in this patient, neither microscopy nor PCR revealed *Plasmodium* spp.)

Expected Result: Negative, no *Plasmodium* antigen detected.

The results are displayed in Table 7:

Table 7: Results for sample 2: *Plasmodium* negative sample. Eligible answers of 125 participants were included.

Reported result	RDT brand						
	Binax	**Palutop**	**Optimal**	**CareStart**	**SD Bioline**	**Ultimed/ Cypress**	**Total (%)**
P. falciparum or mixed infection*			1				1 (0.8)
Absence of control line not reported as invalid*			1		1		2 (1.6)
Negative + comment which is NOT correct†	1	1	1	1			4 (3.2)
Negative + Correct comment	5	4	2	3	5	1	20 (16.0)
Negative	45	21	18	8	5	1	98 (78.4)

*major, † minor and ‡ very minor errors, see definitions in Table 3.

Comment:
Three participants did not answer a final result in terms of interpretation and report to the clinician. Their observations of test lines were correct, but they were not included for analysis. The final denominator consisted of 125 participants.

The single incorrect result ("*P. falciparum* or mixed") for the OptiMAL was confirmed by the raw results of test line observations (strong line intensities reported for both Pf-pLDH and pan-pLDH test lines). This is an unexpected finding, which we assume to be caused by a clerical error (mixing-up of specimen or laboratory data forms) rather than by an analytical one.

Of interest is the absence of the control line in two MRDTs (checked in the raw observations), which were subsequently not reported as invalid results. Invalid test results occur rarely (< 0.5% of samples tested) but consistently (Gillet *et al.*, 2009a; Gillet *et al.*, 2009c; van Dijk *et al.*, 2010) and laboratory staff should be alerted to this phenomenon.

Twenty-four participants added a comment to the report: most comments were valuable adjuncts pointing to the need of repeating the MRDT (and microscopy) in case of a negative result and a persistent suspicion of malaria. However, one comment (cited by two participants) was scored as a minor error: stating that the "Test should be repeated at a next peak of fever" is wrong with regard to the moment of sampling. In case of suspicion of malaria and a negative MRDT, repeat testing should be done. However, as is the case for microscopy, there is no need to await a next peak of fever. Indeed, although there are variations in antigen production during the cycle, there is no clear relation with the concentration of antigens and any particular moment of the cycle, yet the peak of fever (Murray *et al.*, 2008) and a periodic fever pattern does not occur in most of the *P. falciparum* infections. The other comments that were scored as not correct were "Absence of antigen of *P. falciparum* and *P. vivax*" (for the CareStart) and "No current infection". The former comment is linked to an error in the test instructions (see results for sample 10087), the latter is too stringent, as the exclusion power of MRDTs is too low to rule out malaria, in particular the non-*falciparum* species (see also discussion of result 10087).

One might question which strategy to adhere in case of a negative MRDT. We comment on this in the following paragraphs:

What can be the cause of a false negative MRDT in case of P. falciparum?
Under controlled conditions, MRDTs have shown sensitivities close to 100% for the detection of *P. falciparum*, the most life-threatening species. However, as mentioned above, false negative results can occur at low parasite densities (< 100 asexual parasites/µl or < 0.002% of red blood cells parasitized). In such cases, the correct option is the one cited by two participants: repeat testing (both by MRDT and microscopy) after 8 – 12 hours, for up to four consecutive samplings (Farcas *et al.*, 2003; Forney *et al.*, 2001).

Concerns have risen about rare but persistent reports of false negative MRDTs at higher parasite densities. Mostly, they are ascribed to polymorphisms of HRP-2 with the existence of variations that are less likely to be picked up by MRDTs. These variations are geographically confined to the Asia-Pacific and South-American regions (Baker *et al.*, 2005; Gamboa *et al.*, 2010). The impact of these polymorphisms in the scope of travel medicine has not yet been studied.

Besides, the prozone effect is also cited as an explanation for false negative events. The prozone effect (also known as high dose hook phenomenon) is defined as false negative or false low results in immunological reactions, due to an excess of either antigens or antibodies. In MRDTs, the prozone effect has been demonstrated at high parasite densities of *P. falciparum* (> 5% of red blood cells infected) (Gillet *et al.*, 2009b). In these cases, high antigen concentrations will block all available binding sites of both the detection and the capture antibodies, thereby hindering binding of the antigen-detection antibody-conjugate complex to the capture antibody, with failure of signal generation (Figure 10).

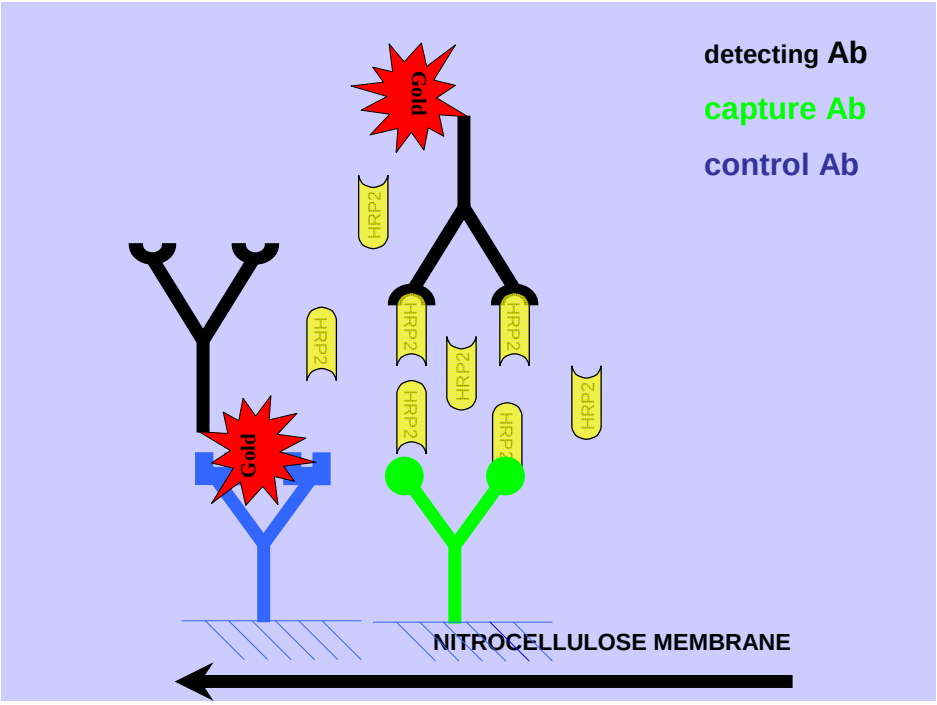


Figure 10: Prozone effect: antigen excess (in this case HRP-2) blocks all available binding sites, thereby preventing the binding between the colloidal gold- conjugated detection antibody (black) and the capture antibody (green).

The prozone effect appears to occur mainly as a partial phenomenon, which means that test line intensities are weak or faint instead of strong. Sometimes however, test lines are not visible at all (Risch *et al.*, 1999). In a recent study, we challenged 20 different MRDTs with a panel of clinical samples with *P. falciparum* hyperparasitaemia. The prozone effect was observed for the HRP-2 test lines in 16/17 MRDTs but not for the *P. falciparum* specific Pf-LDH lines (tested in five MRDTs) and the pan-pLDH and aldolase test lines (Gillet *et al.*, 2009b). In addition, there are the preliminary results of an ongoing field study with our partners in Mozambique that are confirming these observations (Gillet, 2010). The consequences of a false negative HRP-2 result in case of a three- or four-band MRDT is that *P. falciparum* will be erroneously diagnosed as a non-*falciparum* species. In contrast to the false negative results at low parasite densities which can be countered by repeat or serial testing (Farcas *et al.*, 2003; Forney *et al.*, 2001), false negative results in hyperparasitaemia need to be corrected instantly. Microscopy, in parallel to MRDT, will offer a clear hint as in most cases: even a non-experienced laboratory technician will be able to recognize parasites at high densities. Proof of prozone can be obtained by retesting with a diluted sample (for instance a 10 × or even 50 × dilution, in preference with the MRDT kit's buffer): in case of prozone, a test line with (much) higher intensity will be observed (Gillet *et al.*, 2009b). Finally, lab technicians should be trained in correctly reading and interpreting faint and weak test lines and respect the volume of blood to be transferred, as higher volumes of blood not only alter clearing of the MRDT strip but also may increase the risk of the prozone effect (Gillet *et al.*, 2009b).

About false positive MRDT results

False positive MRDTs are rare and there are no prospective data on their frequency. As for the prozone effect, the simple and robust one-step design of MRDTs has its reverse-side: there are no wash steps, reason why non-specific bindings are not removed and interferences may occur. False positive results are particularly observed in patients with the rheumatoid factor and/or in patients with chronic infections, such as toxoplasmosis, hepatitis C, schistosomiasis, tuberculosis and syphilis (Grobusch *et al.*, 2003; Iqbal *et al.*, 2000). Although some studies indicate that HRP-2 based MRDTs are more affected, false positive reactions are observed in all MRDTs (Grobusch *et al.*, 2003). Rheumatoid factor represents auto-antibodies directed against the Fc fragment of IgG molecules. As the rheumatoid factor exhibits considerable immunological heterogeneity, it is assumed that only those molecules with high affinity for the capture antibody will bind, thereby explaining for the fact that only part of the patients with rheumatoid factor apparently show false positive MRDT results (Iqbal *et al.*, 2000).

Another cause of false positive results is the so-called backflow phenomenon. Backflow indicates the appearance of ghost test lines after completion of the test: at warm ambient temperatures, the excess sample with unbound conjugate flows back from the absorption pad to the reaction area and gets deposited on the test band. This is a slow process which occurs after completion (and reading) of the MRDT. Reading beyond the recommended delay is however one of the most common errors among end-users in field settings (Seidahmed *et al.*, 2008). Likewise, backflow is cited as the most common cause of false positive reactions in MRDTs ((Moody, 2002; WHO, 2006), but up to now, its incidence has not been assessed. On the other hand, as immunological reactions are time-dependent, low antigen concentrations may be detected only after long contact times of the antigen during prolonged exposure and for some MRDTs delayed reading is recommended by the manufacturer (WHO, 2009a; WHO, 2010b).

III.4. Results for Sample 10087

Sample 10087:
"(Expatriated) man, 34 years old, suspected of malaria. He has returned from the Democratic Republic of the Congo one week ago. Last year, he has visited India and Haiti. He took prophylaxis."

Diagnosis: *P. vivax*, parasite density 3,251/µl = 0.06%

Expected Result: *P. vivax*, *P. ovale* or *P. malariae* (or "non-*falciparum*" malaria).
(in case of the four-band Palutop: *P. vivax*, mixed infection with *P. ovale* and *P. malariae* not excluded).

The results are displayed in Table 8:

Table 8: Results for sample 3: *P. vivax* sample. Eligible answers of 124 participants were included.

Reported result	RDT brand						
	Binax	**Palutop**	**Optimal**	**CareStart**	**SD Bioline**	**Ultimed/ Cypress**	**Total (%)**
Negative†	51						51 (41.1)
Positive†			1	1	2	1	5 (4.0)
Pan-species†		1	2	2	3		8 (6.5)
P. vivax		23‡		5†			28 (22.6)
P. vivax; P. ovale and *P. malariae* not excluded		2					2 (1.6)
Pan-species, not *P. falciparum*			20	4	6		30 (24.2)

*major, † minor and ‡ very minor errors, see definitions in Table 3.

Comment:
Four laboratories did not report a final result in terms of interpretation and report to the clinician. Their observations of test lines were correct, but they were not included for analysis. The final denominator consisted of 124 participants.

With regard to technical performance, it is clear that the participants using Binax missed the expected result. This observation should be interpreted with caution: the current setting of EQA is not designed as a side-by-side comparison of the different MRDT brands and no conclusions should be drawn with regard to the

comparison of sensitivity of the different MRDT brands used. However, it points to the general problem of the diagnostic sensitivity of MRDTs for the non-*falciparum* species.

What is the diagnostic sensitivity of MRDTs for the non-falciparum species?
Although MRDTs have been extensively evaluated for the diagnosis of *P. falciparum* malaria, their ability to detect the other *Plasmodium* species is less well documented. In addition, due to differences in study design and population, it is difficult to compile all published findings. Most studies have investigated small numbers of the non-*falciparum* species, resulting in low precision but explaining in part for the wide range in reported sensitivities (Marx *et al.*, 2005; Murray *et al.*, 2008). Adding to the difficulties are the evolutions in proprietary compositions of the MRDTs with continuous releases of improved designs but also changing brand names of MRDTs (Hanscheid, 2003). For the Binax NOW kit (most recent generation), compiled sensitivity for the diagnosis of *P. vivax* has been calculated to be 68.9% (Murray *et al.*, 2008). In a population of returned travelers, the Binax NOW displayed sensitivities of 86.7% for pure *P. vivax* samples with false-negative results mainly but not exclusively found among samples with low parasite densities (Farcas *et al.*, 2003). Similar values of sensitivity were obtained in endemic populations in Thailand and Columbia (87.3% and 81.4% respectively) (van den Broek *et al.*, 2006; Wongsrichanalai *et al.*, 2003). In comparison, the OptiMAL-IT which was run side-to-side in the latter study displayed a higher sensitivity for *P. vivax* (91.0%), at the expense of a lower sensitivity for *P. falciparum*. Challenged to a panel of stored samples at ITM, the SD Bioline, Palutop+4 and CareStart showed overall sensitivities for the detection of *P. vivax* of 87.5%, 66.0% and 77.6% respectively ((Maltha *et al.*, 2010; Van der Palen *et al.*, 2009; van Dijk *et al.*, 2009), with, as for the Binax in the current sample, false negatives not limited to low parasite densities.

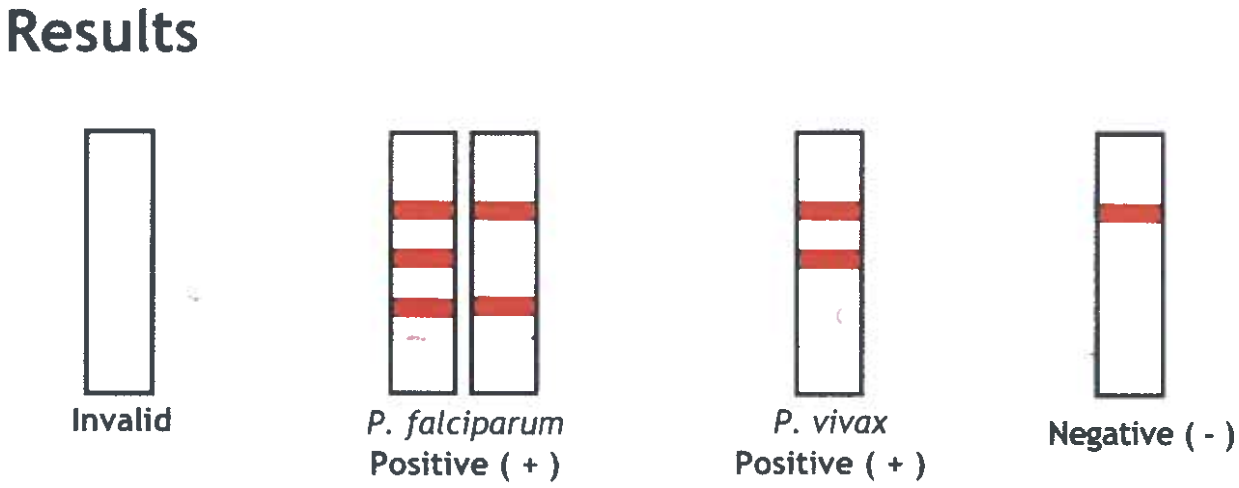
More important than these raw values are the context and diagnostic relevance: even the highest reported sensitivity of 86.7% is not high enough to exclude reliably the diagnosis of *P. vivax*. Further, it has been noted for all MRDT brands that sensitivity declines at parasite densities below 5,000/µl and particularly below 500/µl (Forney *et al.*, 2003; Maltha *et al.*, 2010; Van der Palen *et al.*, 2009; van Dijk *et al.*, 2009; Wongsrichanalai *et al.*, 2007). This is also apparent from the WHO/FIND study, which demonstrated low detection rates among *P. vivax* samples at parasite densities of 200/µl as compared to samples at 2000/µl (WHO, 2009a; WHO, 2010b).

For *P. ovale* and *P. malariae*, the diagnostic sensitivity appears to be even lower. Some so-called pan-specific monoclonal antibodies have a lower affinity for *P. ovale* and *P. malariae* as compared to *P. vivax* (Moody, 2002) and MRDT evaluations have included few data on these species. For the SD Bioline, Palutop and CareStart, we found overall sensitivities for the detection of *P. ovale* of 76.3%, 5.5% and 18.4% respectively; for *P. malariae* the values were 45.2%, 32.0% and 30.4%. As for the *P. vivax* species, there was a sharp decline in sensitivity below the 500/µl threshold of parasite density ((Maltha *et al.*, 2010; Van der Palen *et al.*, 2009; van Dijk *et al.*, 2009). Table 6 shows the parasite densities of the non-*falciparum* species for the samples submitted to ITM, it gives an idea about the number of samples below the 500/µl threshold in a non-endemic setting. Adding to the low detection rate is the fact that pan-pLDH test lines tend to be of lower intensity as compared to the HRP-2 line intensities ((Maltha *et al.*, 2010; Van der Palen *et al.*, 2009; van Dijk *et al.*, 2009).

Apart from this analytical issue, there were again some problems of interpretation and reporting. Reports such as "pan-species" or simply "positive" (approximately 11% of participants) are hiding the information that *P. falciparum* is not involved. These reports should be replaced by more informative ones, such as: "non-*falciparum* species" or "*P. vivax*, *P. ovale* and *P. malariae*", in case of a three-band MRDT and in case of four-band MRDTs "*P. vivax*, mixed infection with *P. ovale*/*P. malariae* not excluded". Of course, the chance of finding a mixed *P. vivax*/*P. ovale* infection will be extremely low because *P. ovale* is geographically confined to Central- and West-Africa, where *P. vivax* is very rare (Culleton *et al.*, 2008).

For the interpretation of Palutop, we refer to Figure 3, explaining for the very minor error of the "*P. vivax*" report. Of note, there were five participants using CareStart who reported "*P. vivax*" despite the fact that the kit used does not include a *P. vivax*-targeted antibody. We assume the reason of this error to be borne into the package insert. In fact, CareStart is presently marketed both as individually wrapped test kits ("Single Kits") and laboratory kits (Maltha *et al.*, 2010). When it comes to the interpretation of a single pan-pLDH line, the package inserts of both the laboratory kit and "Single kit" have errors: the former mentions "pan-species" whereas the latter mentions "*P. vivax*" (Figure 11).

Figure 11: Package insert of CareStart "Single Kit", test interpretation stating "*P. vivax*" instead of "non-falciparum species" when a single pan-pLDH line is visible.



III.5. Results of the Questionnaire

Exposure to malaria-positive samples

Table 9 depicts the number of requests for malaria diagnosis processed by each laboratory in 2009, matched to the number of laboratory staff. Assuming a 5-10% positivity rate for patients suspected of malaria, it is clear that laboratory technicians have little exposure to malaria-positive slides: even in those laboratories with a high number of requests, the critical volume is diluted by the high number of laboratory staff participating in the diagnosis. This is comparable to the situation in other countries, with about half of laboratories in the U.K. carrying out less than 100 malaria requests each year and about 10% of laboratories fewer than 10 (Chilton *et al.*, 2006). In France, about 60% of 3.300 surveyed laboratories declared to have seen at least one malaria case in the previous year, and only 5% make the diagnosis of malaria five times or more per year (Moulin & Gendrel, 2009).

In line with the low number of requests was the reported number of MRDTs processed in 2009 as reported: almost 60% of the participants (71/125, 56.8%) had processed 20 tests or fewer, another third (44/125, 35.2%) had processed between 20 and 100 tests. The technique of immunochromatography allows for an extended stability of the strips, and MRDT kits are complying with the WHO recommendation of a shelf-life of at least 15 months after delivery (WHO, 2004).

Table 9: Cross tabulation of the numbers of laboratory staff involved in malaria diagnosis versus the numbers of requests for malaria diagnosis in 2009.

Numbers of requests for malaria diagnosis in 2009	**Numbers of laboratory staff performing malaria diagnosis**				
	0-5	**6-10**	**11-20**	**> 20**	**Total**
0-10	4	8	16	3	31
11-20	11	7	11	1	30
21-100	4	16	17	12	49
101-500	4	4	5	1	14
> 500		1			1
Total	23	36	49	17	125

Impact on the diagnosis of malaria

More than three quarters (97/120, 80.8%) of participants replied that MRDTs had improved the diagnosis of malaria in their setting. The remaining participants answered that there had been no influence.

In a previous survey in the UK, only 12.6% out of 305 respondents stated that MRDTs had revised their malaria diagnosis. Although the meaning and phrasing of the latter question were different ("revised" versus "improved"), the difference in numbers is striking. It may be attributed to a growing experience with MRDTs in clinical laboratories.

Ease of use

Ease of use as expressed on a scale from 0 to 10 is listed for each of the MRDT brands in Table 10. Although the median scores did not differ too much and differences did not reach statistical significance, there was a wider range with a tendency to lower scores for Binax, and, to a lesser extent, OptiMAL. This is not unexpectedly, as these kits include more steps than the more recently released one-step kits such as CareStart and SD Bioline.

Table 10: Ease of use of the different MRDTs expressed as a score from 0 to 10.

MRDT brand	Numbers of laboratories using this kit	Median Score	Range
Binax	52	8	2-10
OptiMAL	23	8	6-9
Palutop	25	9	7-10
CareStart	12	9	8-10
SD Bioline	11	9	8-10

In our experience as well as in that of others (Luchavez *et al.*, 2007; McMorrow *et al.*, 2008), particular problems can arise with the use of the transfer device: without having processed high numbers of samples, it may be difficult to master the MRDT kit's loops, straws or capillary tubes, which are frequently small and not user-friendly. The volume of blood used to run the MRDT is critical: an excess of blood may increase the risk and/or the intensity of prozone effect or may mask a faint line due to a bad clearance of the strip, while a shortage of blood may decrease the sensitivity of the test. Even after an adequate training, there is high variability in blood volume transferred by the kit's devices. The straw and loop usually transfer volumes greater than intended, while the glass capillary tube and the plastic pipette transfer less volume than intended (Luchavez *et al.*, 2007). Therefore we recommend the use of regular transfer pipettes rather than the manufacturer's transfer devices.

Idea of the sensitivity

Most respondents have a clear idea about the sensitivity of the MRDT for the diagnosis of *P. falciparum*. Some respondents misunderstood the question, *i.e*. they interpreted the question as being referred to their own laboratory data; others reported the analytical sensitivity (detection limit) and not the diagnostic sensitivity. Most data were based on the product information in the kit's instructions. In contrast to a previous survey (Chilton *et al.*, 2006), the range of sensitivity reported was small and the values were mostly accurate.

Diagnostic strategy of MRDTs

In view of their strengths, MRDTs are a valuable adjunct for malaria diagnosis and should – in our opinion – be used together with microscopy in all cases when the diagnosis of malaria is suspected. Conversely, their limitations do not justify MRDTs as the unique tool for diagnosis: microscopy is needed to recover diagnosis that may be missed by MRDTs (low parasite densities, prozone effect, non-*falciparum* species), to determine parasite density, and to recognize stages and prognostic indicators (hemozoin and *P. falciparum* schizonts) (Table 2).

Of particular interest was the strategy for malaria diagnosis and the place of microscopy and MRDTs reported by the participants. A total of 125 participants gave eligible answers on their strategy during opening hours, among them there were 113 who offered malaria diagnosis outside opening hours. Tables 11 and 12 list the diagnostic strategies during and after opening hours (weekend and night shifts). The vast majority (94.4%) of participants used MRDTs as a complement or adjunct to microscopy during opening hours, but outside opening hours only 62.8% did so. Moreover, outside opening hours, 31.1% of them relied on RDTs as the primary (4.4%) or the single tool (25.7%) for malaria diagnosis. Of note, five participants added that they do not perform MRDTs on follow-up samples: this is a correct policy, as HRP-2 may persist in the circulation for up to several weeks. Aldolase and pLDH depend on living parasites and rapidly decline after start of correct treatment, but their use is limited because they are also expressed by gametocytes (Mueller *et al.*, 2007). By consequence, monitoring of treatment efficacy should be done by microscopy only.

Table 11: Strategy of malaria diagnosis during opening hours as reported by 125 participants.

Diagnostic strategy of malaria during opening hours	**Numbers of participants (%)**
Microscopy + always MRDT	99 (79.2)
Microscopy + MRDT for confirmation	18 (14.4)
Microscopy + MRDT if requested by the clinician	2 (1.6)†
Microscopy and/or MRDT, depending on the request by the clinician	3 (2.4)†
MRDT, if positive or in case of doubt: + microscopy	3 (2.4)*

*major and † minor errors, see text.

Table 12: Strategy of malaria diagnosis outside opening hours as reported by 113 participants.

Diagnostic strategy of malaria outside opening hours	Numbers of participants (%)
Microscopy + always MRDT	63 (55.8)
Microscopy + MRDT for confirmation	8 (7.1)
Microscopy + MRDT if requested by the clinician	2 (1.8)†
Microscopy alone	2 (1.8)†
Microscopy and/or MRDT, depending on the request by the clinician	3 (2.7)†
MRDT: if MRDT positive, microscopy is done instantly; if MRDT is negative, microscopy is done the next day	5 (4.4)*
MRDT + microscopy next day	1 (0.9)*
MRDT: if positive or in case of doubt: + microscopy	16 (14.2)*
MRDT alone, no microscopy	13 (11.5)*

*major and † minor errors, see text

We considered the policy of leaving the decision of using the MRDT to the attending clinician as a minor error, at the risk of not exploiting the possible information generated by the MRDT. In line, the policy of leaving the choice between microscopy and MRDT to the clinician was considered a minor error: here, the risk that he or she might choose uniquely for the MRDT is not countered and may lead to a missed diagnosis.

The policy of leaving the decision of leaving the choice of MRDT or microscopy to the attending clinician may result in not performing the MRDT (thereby not exploiting possible information generated by the MRDT) or not performing microscopy. It is probably is related to the reimbursement system of medical costs in Belgium: national health insurance reimburses only laboratory analyses that are explicitly requested by the clinician. Hospital-based diagnostic and treatment algorithms can guide the choice and priorities of laboratory tests, but for the individual patient however, the ultimate request of MRDT, microscopy or both depends on the clinician's decision. A reimbursement policy that links the request of MRDTs to a complementary microscopic analysis would be beneficial, as it would not only reduce the request of MRDTs without microscopy but also counter the policy of not performing microscopy outside office hours.

The strategy of relying on MRDTs as the unique diagnostic tool was considered a major error, whether microscopy was postponed till the next day (in the outside opening hours strategy) or simply not performed at all. Such a strategy concerned a small minority of participants during opening hours (2.4%) but nearly one

third (31.1%) outside opening hours, who relied on RDTs as the primary (4.4%) or the single tool (25.7%) for malaria diagnosis (Tables 11 and 12). In the 2006 U.K.-survey, a similar tendency was observed: less than 5% of 327 surveyed laboratories used exclusively a MRDT during opening hours, versus 15 – 20% outside opening hours (Chilton *et al.*, 2006). The extent of the potential risks as a result of this strategy should not be underestimated: especially since, according to a survey in Portugal, about half of the patients suspected of malaria present outside opening hours, and they account for 60% of diagnoses (Hanscheid, 2003). It is important to ascertain reliable diagnosis of malaria during and outside office hours, and competent microscopy should be in reach at all times.

IV Summary: the place of Malaria Rapid Diagnostic Tests

From the present EQA session, it is clear that MRDTs are an essential part of malaria diagnosis in most diagnostic laboratories in Belgium. Apart from an occasional disregarded invalid test, the MRDTs' analytical performances reached those published by reference settings. Improvements could be made for the pre- and post-analytical phases: reliance on MRDTs without simultaneous microscopy (a common practice outside opening hours) should be avoided, and reports of results should include information about the presence or absence of *P. falciparum* whenever possible. Manufacturers should be encouraged to revise and adapt their instructions where needed, especially with regard to test line interpretations.

MRDTs are a valuable adjunct to microscopy in the diagnosis of malaria. Table 13 gives the summary of their added value and Table 14 lists the most relevant "Do's and Don'ts" for MRDTs when applied in the non-endemic setting.

Laboratories are invited to send positive or doubtful samples for second opinion and confirmation to the Institute of Tropical Medicine. We refer to the instructions on the request forms available on-line (see Table 14).

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Table 13: What MRDTs can add to the diagnosis of malaria.

Requirements for malaria diagnosis (see Table 2)	**Contribution of MRDTs**	**Comments**
Timely confirmation or exclusion of the diagnosis of malaria with prompt referral in case of doubt	Considerably helpful in the diagnosis of malaria	Excellent sensitivity, especially for *P. falciparum* > 100 parasites/µl False-negatives for *P. falciparum* at low parasite densities (<100/µl), occasionally above Certain HPR-2 mutations may not be picked-up
	Do not rule out malaria in a confident way (microscopy needed as well)	Prozone effect is rare but occurs, particularly in HRP-2 based MRDTs Only of moderate help for diagnosis of *P. vivax* and of little help for *P. ovale* and *P. malariae*
Distinction between *P. falciparum* (possible life-threatening) and the non-*falciparum* species	Of considerable help in the identification of *P. falciparum*	Mixed infections are rare but not excluded if *P. falciparum*- and pan-species antigen lines are present
Assessment of parasite densities, in particular recognition of critical values (>2% of red blood cells infected)	Of no help	Line intensities are indicative for parasite density but here is a very large overlap Unique HRP-2 line may point to low parasite density
Recognition of *P. falciparum* stages and hemozoin	Of no help	

Table 14: Do's and don'ts of MRDTs.

Do's and don'ts in the use of MRDTs	**Comments**
Check the control line - repeat MRDT if control line is not visible	Absence of control line means invalid test and no conclusion can be drawn
Do not store MRDTs in the freezer	Freezing will destroy colloidal gold
Do not read before or beyond the recommended reading time	Reading to early may cause false-negative results Waiting too long may cause false-positive results
Respect the correct volume (you may use a pipette instead of the transfer device)	Too little blood may cause false-negative results Too much blood will cause decreased clearance of the strip, hindering reading Too much blood may increase the risk/intensity of prozone effect
Consider a faint line also as a positive line	Any visible line is a positive line
Repeat a negative MRDT in case of suspicion of malaria	Repeat after 8-12 hours for a successive 4 times over 36 hours to rule out malaria
Use a transfer pipette	Transfer devices of MRDT kits tend to be small and somewhat difficult to manipulate
Do not use MRDTs for treatment follow-up	HRP-2 based MRDTs remain positive for weeks after *P. falciparum* infection, gametocytes express pLDH and aldolase
Always combine MRDT with microscopy	See Table 13
Send any positive or doubtful sample to the reference laboratory for confirmation	See request form and instructions on: https://www.iph.fgov.be/epidemio/epinl/plabnl/N_Plasmodium.pdf https://www.iph.fgov.be/epidemio/epifr/plabfr/F_Plasmodium.pdf

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