Chapter 8

General Discussion
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The severe effects of malaria on pregnant women and their infants make early diagnosis and subsequent safe and effective treatment of great importance. Different diagnostic tools such as microscopy and rapid diagnostic tests (RDTs), which have been shown to work well for uncomplicated malaria, are being used for the diagnosis of malaria in pregnancy as well. Nevertheless, research on the diagnostic accuracy of these tests in this population is limited. This thesis aimed to provide an evidence-based conclusion on the accuracy of diagnostic tests for the diagnosis of malaria in pregnant women. A second objective was to develop new antibodies for diagnostic tests for malaria and subsequently evaluate them in pregnant women.

8.1 Principal findings

The findings of the research described in this thesis suggest that rapid diagnostic tests (RDTs) and molecular techniques such as polymerase chain reaction (PCR) have good diagnostic performance characteristics to serve as alternatives to microscopy for the diagnosis of malaria in pregnancy. The review described in Chapter two has shown that in particular histidine-rich protein 2 (HRP2)-based RDTs and PCR show good accuracy compared to microscopy. However, a lack of research determining the accuracy of RDTs and PCR for the diagnosis of placental malaria with placental histology as reference test was found. In the field evaluations described in Chapters six and seven, high malaria prevalence was found amongst asymptomatic women during pregnancy and at delivery. In these studies both PCR and the HRP2-based RDT showed good accuracies in peripheral blood compared to microscopy of peripheral blood and impression smears. The \textit{Plasmodium} lactate dehydrogenase (pLDH) based test performed less well, but much better than expected based on the evaluation of pLDH-based tests reported in Chapter two. The downside of the HRP2-based RDTs is the significantly longer positivity of the test after treatment compared to other tests due to persistence of the antigen (Chapter 5). A comparison with histology could unfortunately not yet be completed before submission of this thesis.

A start has been made towards the development of new diagnostic tests. Novel monoclonal antibodies (mAbs) were developed and characterized against the \textit{Plasmodium} antigen targets dihydrofolate reductase-thymidylate synthase (DHFR-TS), heme detoxification protein (HDP) and glutamate rich protein (GLURP). It has been shown that several of the developed antibodies react with good affinity and detection limit with the recombinant antigens and \textit{Plasmodium falciparum} and \textit{Plasmodium vivax} isolates and the antibodies show much potential for further development in diagnostic assays. These antigens were less persistent in pregnant women after treatment than HRP2. Subse-
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Quently, several antibodies were evaluated in an ELISA setup to detect genetically divers *P. falciparum* cultured isolates, *P. vivax* from patient samples and malaria parasites in the samples from the field evaluations. However, in this setup the ELISA did not have sufficient accuracy for the diagnosis of malaria in these samples.

### 8.2 Epidemiological and clinical setting

The conclusions of the research described in this thesis are mainly applicable to high transmission regions in sub-Saharan Africa. In the review of published diagnostic accuracy (Chapter two), 85% of the included studies were conducted in sub-Saharan Africa and the field evaluations that are described in Chapters five, six and seven are from a high transmission area in that part of the world as well. Only a few studies have been conducted that evaluate diagnostics in the Asian-Pacific region. Malaria prevalence in these areas, however, is lower, resulting in more time and costs needed to perform such an evaluation. In line with this observation, the diagnostic accuracies are most applicable to *P. falciparum* infections, being the predominant cause of malaria infections in sub-Saharan Africa.

The field evaluations of the diagnostic tests described in Chapter six and seven were performed among mostly non-febrile and asymptomatic pregnant women in Burkina Faso during their regular antenatal care (ANC) visits or at delivery. Also in the majority of studies reported in the systematic review, the patients were recruited during ANC visits or cross-sectionally at delivery. Most of the pregnant women were asymptomatic, which is to be expected since most studies were performed in high transmission areas where infection often occurs without any symptoms due to developed immunity. The results of this thesis are therefore most applicable to asymptomatic pregnant women infected with malaria parasites. However, if the studies recruiting symptomatic patients that were reported in Chapter two [120, 126, 137] are compared to studies with asymptomatic women, no clear difference in accuracy can be seen.

### 8.3 Comparison with literature

Diagnostic accuracy of tests for the diagnosis of malaria in pregnancy has not been analysed in a meta-analysis before. In a previous report from Uneke et al. a comparison was made of the prevalence of placental malaria estimated by different tests in different studies [57]. The limitations of that study are that even if the prevalence detected by two tests is similar, this does not mean that the same cases are being detected by both tests. Nevertheless, the conclusions
of that report and this thesis are broadly similar. Uneke et al. report that a higher prevalence was found for histology than placental blood microscopy and placental blood microscopy in turn reported to have a higher prevalence than peripheral microscopy. Higher prevalence was found for PCR than RDT and for both tests prevalence was higher than with microscopy. In this report no studies were reported that compared RDT and PCR to histology [57].

A review on malaria in pregnancy in the Asia-Pacific region has been presented with a nice overview of published and unpublished data on diagnostic tests for malaria in pregnancy in that region, but the authors did not perform a meta-analysis [197]. Very few studies on placental histology and only one study evaluating RDTs were included in the review by Rijken et al. [137]. This included RDT study was discussed in the systematic review described in Chapter 2 as well. In the review by Rijken et al., however, it was concluded that more cases were detected by RDTs than by microscopy and that this might have been due to persistence of the HRP2 antigen [197]. Although few reports were available of PCR diagnostic assessment of malaria in pregnancy in the Asia-Pacific region, PCR was reported to detect more cases of P. vivax and P. falciparum infection than microscopy or RDTs, which is very similar to the results presented in this thesis [197].

8.4 Summary values of diagnostic accuracy

When the diagnostic accuracies are compared that were obtained in the field evaluations in this thesis to the summary accuracies presented in Chapter two, quite similar values for PCR and RDTs are found compared to microscopy of peripheral blood, although specificity was a little lower for both RDTs and microscopy in Chapter six. The pLDH based test used in these evaluations had significantly higher sensitivity compared to the studies described in the meta-analysis. The sensitivity of an RDT depends on the local conditions and will vary between populations with different levels of transmission. However, the WHO evaluated RDT products and reported which RDTs are more likely to provide higher sensitivity in the field [62, 63]. In the second WHO-report the performance characteristics of the Advantage Malcard RDT, used in this thesis, was shown to be superior to the performances of the pLDH RDTs used in the other studies, which might explain the variation in sensitivity [63].

The 2×2 tables of the tests evaluated in this thesis can be added to the analyses performed in Chapter two to obtain new summary estimates of sensitivity and specificity. In addition, the MEDLINE search was repeated (9 Jan 2012) to include any published articles since the last search. Six articles were found that were of interest based on title and abstract [198–203]. Unfortunately, one of them could not be accessed and therefore could not be
Figure 8.1 Summary ROC plot of sensitivity and specificity of microscopy and RDT of peripheral and placental blood and PCR of placental blood with placental histology as a reference test.

The sensitivity of a test is plotted against 1-specificity, allowing comparison of both parameters at the same time for multiple tests. The rectangles diamonds and circles represent individual studies and size of the rectangles/diamonds/circles is proportional to the number of patients included in the study. The thick round spots are the summary estimates of sensitivity and specificity and the dotted ellipses around the spots represent the 95% confidence intervals around the summary estimates. Black: RDT of peripheral blood; Red: RDT of placental blood; Green: peripheral blood microscopy; Blue: placental blood microscopy; Yellow: PCR of placental blood. The reference test used to determine the plotted accuracies in this figure is placental histology.
included, this was however a comparison between microscopy of placental and peripheral blood [203]. Two of the retrieved studies described the evaluation of variations on traditional microscopy and were not of interest for this analysis, because no RDTs or PCR were evaluated [200, 202]. The three studies that did evaluate an RDT or PCR did so against histology as a reference test [198, 199, 201]. Unfortunately, the histology results of the evaluations presented in Chapter seven are not yet available upon submission of this thesis; therefore there are still not enough studies to perform meta-analysis with histology as a reference test. The studies comparing PCR or RDTs to histology are plotted in Figure 8.1 together with the retrieved results for microscopy presented in Chapter two. The RDTs with peripheral or placental blood seem to have higher sensitivity than microscopy of peripheral blood, and the RDTs with peripheral blood have similar specificities as microscopy of peripheral blood (Figure 8.1). The two studies presenting PCR of placental blood both have low accuracy (Figure 8.1). Studies that compared PCR in peripheral blood to placental histology were not found.

As an alternative, the accuracies of RDTs and PCR can be determined with microscopy of placental blood as a reference. With the results from the evaluation in Chapter seven, the accuracies of RDTs and PCR with peripheral and placental blood could now be analysed separately. This new analysis shows that the RDTs with peripheral blood have a lower summary specificity (88% [80-93 CI]) than RDTs of placental blood (95% [90-98 CI]), although there is quite some overlap in confidence region (Figure 8.2 and Table 8.1). The summary sensitivity of RDTs with peripheral blood is slightly higher than placental blood, although not significantly (Figure 8.2 and Table 8.1). The summary estimates of sensitivity and specificity of PCR of peripheral and placental blood are very similar, although specificity is slightly lower in peripheral blood (Figure 8.2 and Table 8.1). The summary specificities of RDTs of peripheral blood and PCR are significantly lower than that of peripheral microscopy. The summary sensitivities of RDTs and PCR, however, are higher than that of peripheral microscopy (Figure 8.2 and Table 8.1).

When assessing accuracy with placental blood microscopy as reference test, however, bias might be introduced by the method in which the blood slide was prepared, since a big difference in accuracy was found in Chapter seven between microscopical slide examination of placental blood that was pooled in an incision in the placenta and microscopical examination of placental tissue from the same incision site. In the review in Chapter two there are too few tests to compare the different methods to placental histology in a meta-analysis. Nevertheless, if we compare the accuracy of peripheral microscopy to different methods of preparing placental slides, (grouped in blood collected from incisions and impression or swabbed or scrapped smears), a significantly
8.4. SUMMARY VALUES OF DIAGNOSTIC ACCURACY

Figure 8.2 Summary ROC plot of sensitivity and specificity of RDT and PCR of peripheral and placental blood and microscopy of peripheral blood with placental blood microscopy as reference test.

The sensitivity of a test is plotted against 1-specificity, allowing comparison of both parameters at the same time for multiple tests. The squares, diamonds and open circles represent individual studies and the size of these figures is proportional to the number of participants in the study. The thick round spots are the summary estimates of sensitivity and specificity and the dotted ellipses around the spots represent the 95% confidence intervals around the summary estimates. Black (rectangles): RDTs of peripheral blood; Red (diamonds): PCR of peripheral blood; Green (circles): PCR of placental blood; Blue (rectangles): peripheral blood microscopy; Yellow (diamonds): RDTs of placental blood. The reference test used to determine the plotted accuracies in this figure is placental blood microscopy.
lower sensitivity for peripheral microscopy is found compared to tissue smears (51.7% [38 - 65 CI]) than compared to blood smears (77.8% [68 - 85 CI]) (Figure 8.3). Although this is no direct evidence that tissue smears are better at detecting placental malaria infection than placental blood smears, it does mean that we should be careful when considering the results with placental blood microscopy as reference test, and better comparisons are required.

Many studies evaluating diagnostics have been performed during pregnancy and not at delivery, and therefore peripheral blood microscopy is often used as a reference test. It has been shown that, compared to histology, peripheral microscopy fails to detect many placental infections (Chapter 2). Nevertheless, some conclusions can be drawn from these comparisons. With the new data from the evaluations presented in this thesis, it is now possible
Figure 8.3 Summary ROC plot of sensitivity and specificity of microscopy of peripheral blood with placental blood microscopy as reference test.

The sensitivity of a test is plotted against 1-specificity, allowing comparison of both parameters at the same time for multiple tests. The squares and diamonds represent individual studies and the size of these figures is proportional to the number of participants in the study. The thick round spots are the summary estimates of sensitivity and specificity and the dotted ellipses around the spots represent the 95% confidence intervals around the summary estimates. Black (rectangles): peripheral blood microscopy with placental blood smears as reference test; Red (diamonds): peripheral blood microscopy with placental tissue smears as reference test.
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to perform a subgroup analysis between pLDH-based tests and HRP2-based tests compared to microscopy of peripheral blood. There are four studies with pLDH-based tests that can be included in the meta-analysis, but there is a lot of variance in sensitivity, as explained earlier. In general, HRP2-based studies show consistently higher sensitivity than pLDH-based tests; although specificity is consistently lower (Figure 8.4 and Table 8.1). It was observed as well that accuracy of HRP2-based RDTs is similar to the accuracy of PCR, at least compared to peripheral blood microscopy, with only a slightly higher specificity for HRP2-based RDTs (Figure 8.4 and Table 8.1).

8.5 Implications for malaria control in pregnancy

As explained in Chapter one, there are differences in the presentation of malaria between high and low transmission regions that can have an effect on diagnostic accuracy, the choice of tool and the diagnostic pathway (using a single test, or a combination of tests). In low or unstable transmission areas, malaria in pregnancy usually presents as a symptomatic, severe disease that can result in death of mother and foetus. In contrast, in areas of high (stable) malaria transmission, malaria infection rarely results in symptomatic disease during pregnancy and the main impact is malaria-related anaemia in the mother and low birth weight and stillbirth in infants. It is to be expected that the parasite density is lower in pregnant women in high transmission areas than in low transmission areas, because it is being controlled by their immune system, especially in the case of the parasites in the peripheral circulation. Therefore, it is important to use tests in these areas that can detect low parasite densities and sequestered parasites, such as PCR and HRP2-based RDTs. In low transmission areas parasite densities are higher and peripheral parasites are more frequent, but a larger proportion of infections are non-\textit{falciparum}. Therefore, the detection limit of the test is less important, but the capability of the test to distinguish between species becomes more important. In this case PCR, microscopy or tests with a combination of PAN detection by pLDH and \textit{P. falciparum} detection by HRP2 might be more suitable.

In Chapter six and seven we have shown that quite a large proportion of pregnant women in the study area in Burkina Faso had low density malaria parasitaemia detected by HRP2-based RDT and PCR. These women were tested during regular ANC visits or at delivery and the majority did not present with any illness or symptoms. Many of these women were positive, despite the implementation of intermittent preventive treatment (IPTp) in the region. This could indicate that although malaria control measures such as IPTp and the distribution of insecticide treated nets (ITNs) are implemented, malaria infection in these women is still quite prevalent. Possible contributing fac-
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Figure 8.4 Summary ROC plot of sensitivity and specificity of RDT and PCR with peripheral blood with peripheral blood microscopy as reference test.

The sensitivity of a test is plotted against 1-specificity, allowing comparison of both parameters at the same time for multiple tests. The rectangles, diamonds and circles represent individual studies and size of these figures is proportional to the number of participants in the study. The thick round spots are the summary estimates of sensitivity and specificity for the different test types and the dotted ellipses around the spots represent the 95% confidence intervals around the summary estimates. Black (rectangles): PCR; Red (diamonds): HRP2 based RDTs only; Green (circles): pLDH based RDTs only. The reference test used to determine the plotted accuracies in this figure is peripheral blood microscopy.
tors might be a poor IPTp coverage or compliance (of sufficient IPTp doses) or a growing resistance against sulphadoxine-pyrimethamine (SP), the drug of choice for IPTp. In 2007 an evaluation was conducted in Burkina Faso to assess the level of SP resistance in malaria parasites, and this study reported that although SP is still relatively effective in Burkina Faso, a considerable number of mutations were found that have been related to the development of SP resistance [204].

However, another issue related to IPTp use that affects diagnostic accuracy is the relatively slow parasite clearance and increased gametocyte induction by SP [188, 193]. The study described in Chapter five has shown that especially HRP2 can persist for a long period after ACT treatment. This makes it difficult to interpret a positive HRP2-based RDT result between IPTp doses, since it can mean the presence of a true infection, the presence of only gametocytes or the test is false positive due to HRP2 persistence after parasite clearance and might thus also partially explain the high prevalence by the HRP2-RDT in Chapters 6 and 7, although the prevalence by PCR was also high. This persistence has important consequences for countries where besides IPTp, RDTs based on HRP2 are used for diagnosis, such as Burkina Faso that is considering to implement HRP2-based tests as routine diagnostics for malaria in pregnancy, and this can lead to unnecessary treatment of the pregnant women for malaria and possible under-recognition of other diseases. It has been shown in this thesis that although microscopy and pLDH-based RDTs do not have these problems with antigen persistence, these tests have insufficient accuracy for the diagnosis of malaria in pregnancy in high transmission areas. Until better tests become available, HRP2-based RDTs might best be used. Although there will be some over treatment of pregnant women with persistent antigen, this will not be a big problem given the acceptable safety profile that the ACTs have in the 2nd and 3rd trimester. Secondly, the long-acting component of the ACT has the benefit of acting as a post-treatment prophylactic effect.

In other areas, mainly those with low or seasonal transmission, screening and subsequent treatment of women during ANC visits has been proposed and is being used [44, 56]. Using IPTp in low transmission areas might result in a large proportion of pregnant women receiving SP unnecessarily, and this can be prevented by these screen-and-treat strategies. An essential element of this strategy is good accuracy of the test. Moreover, it requires an affordable and quick diagnostic tool, such as an RDT. At the Shoklo Malaria Research Unit clinics on the Thai-Burmese border, for example, pregnant women receive weekly antenatal care, including malaria screening with microscopy [44, 55]. This early detection and treatment with ACTs has led to a reduction of placental malaria cases and placental malaria has been confined to pregnancies.
with concurrent maternal peripheral parasitaemia [44, 197, 205]. RDTs pose a practical and accurate alternative to microscopy for the diagnosis of pregnant women, but in these screen-and-treat strategies antigen persistence will be a problem as well, especially if the subsequent screening is performed within a short time span.

8.6 Unanswered questions and future research

Even though this thesis has shown good performance of RDTs and PCR compared to microscopy of peripheral and placental blood to diagnose malaria infection in blood of pregnant women, more data are needed that compare these tests to histological examination of the placenta. Histology results of the evaluation in Chapter seven will become available and will be published along with the results described in that Chapter. Nevertheless, more well-performed RDT-evaluations on peripheral blood are needed to enable an evidence based conclusion on their accuracy. This is especially true for pLDH RDTs, since such a large variation in accuracies has been found. Studies designed to evaluate RDTs in pregnant women are advised to choose RDTs with good overall test characteristics, as determined by the WHO/FIND RDT evaluation, since a test with poor characteristics with those samples, will never perform well in pregnant women either, and this might give the false impression that none of the RDTs would work well in that population [62, 63]. PCR is positive in many more cases than any other test and seems to be a sensitive test for the diagnosis of malaria in pregnant women, although few studies are available comparing PCR to histology. Development of advanced technologies using the amplification of nucleic acids is ongoing and more and more technologies are becoming available for malaria that dramatically speed-up the process and simplify outcome analysis [206–210]. Soon, tests based on nucleic acid detection will become commercially available that are quick, easy-to-use and not too expensive, and they might be implemented for routine use in the future. Therefore, in addition to RDT-evaluations, more studies are urgently required that evaluate PCR with peripheral blood and compare it to histology.

As mentioned above, the outcomes of this thesis apply mainly to sub-Saharan Africa and *P. falciparum* infections. More studies should be performed in other areas such as South America and the Asian-Pacific region, where many malaria infections occur as well. Most pregnancies at risk are found in the Asian-Pacific region, making it an important region for diagnostic test use, especially if screen and treat strategies are implemented [11, 23, 56]. Due to the potential pathological differences between *P. falciparum* and *P. vivax* infection in pregnancy, a difference in diagnostic accuracy of the var-
ious tests is expected between the species and more studies should aim at evaluating diagnostics in *P. vivax*-infected pregnant women in the future.

There is an obvious need for new easy-to-use diagnostics for the diagnosis of malaria in pregnancy with a better detection threshold and accuracy than pLDH-based RDTs and microscopy, but fewer problems in terms of antigen persistence as HRP2-based RDTs. Nucleic acid based assays might offer a solution, but the antibodies developed against the antigens DHFR-TS, HDP and GLURP show much potential for further development in diagnostic assays as well. It has been shown that several of the developed antibodies react with good affinity and sensitivity with the (recombinant) antigens in solution. The background with whole blood in ELISAs, however, is still an issue that hampers test development, as well as finding antibody couples that show no background reaction with uninfected whole blood. If tests with these antibodies can be optimized to more accurate assays than presented in this thesis, they could be a good alternative for malaria diagnosis in pregnant and non-pregnant individuals.