AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

# Absence of Association between Polymorphisms in the RING E3 Ubiquitin Protein Ligase Gene and *Ex Vivo* Susceptibility to Conventional Antimalarial Drugs in *Plasmodium falciparum* Isolates from Dakar, Senegal

**Mathieu Gendrot,[a,b] Bécaye Fall,[c] Marylin Madamet,[b,d,e] Mansour Fall,[f] Khalifa Ababacar Wade,[g] Rémy Amalvict,[b,d,e] Aminata Nakoulima,[h] Nicolas Benoit,[b,d,e] Silman Diawara,[c] Yaya Diémé,[c] Bakary Diatta,[g,i] Boubacar Wade,[i] Bruno Pradines[a,b,c,e]**

Unité de Parasitologie et d'Entomologie, Département des Maladies Infectieuses, Institut de Recherche Biomédicale des Armées, Brétigny sur Orge, France[a]; Aix Marseille Université, Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, UM 63, CNRS 7278, IRD 198, Inserm 1095, Marseille, France[b]; Laboratoire d'étude de la chimiosensibilité du paludisme, Fédération des laboratoires, Hôpital Principal de Dakar, Dakar, Sénégal[c]; Equipe résidente de recherche en infectiologie tropicale, Institut de Recherche Biomédicale des Armées, Hôpital d'instruction des armées, Marseille, France[d]; Centre national de référence du Paludisme, Marseille, France[e]; Service de Réanimation Médicale, Hôpital Principal de Dakar, Dakar, Sénégal[f]; Service des Urgences, Hôpital Principal de Dakar, Dakar, Sénégal[g]; Service de Pédiatrie, Hôpital Principal de Dakar, Dakar, Sénégal[h]; Chefferie, Hôpital Principal de Dakar, Dakar, Sénégal[i]

**The RING E3 ubiquitin protein ligase is crucial for facilitating the transfer of ubiquitin. The only polymorphism identified in the E3 ubiquitin protein ligase gene was the D113N mutation (62.5%) but was not significantly associated with the 50% inhibitory concentration ($IC_{50}$) of conventional antimalarial drugs. However, some mutated isolates (D113N) present a trend of reduced susceptibility to piperaquine ($P = 0.0938$). To evaluate the association of D113N polymorphism with susceptibility to antimalarials, more isolates are necessary.**

Malaria resistance to most antimalarial drugs has developed in southeast Asia and has spread to Africa. The World Health Organization (WHO) has recommended artemisinin-based combination therapy (ACT) as the first-line treatment for malaria since 2005. As recently described in southeast Asia, the emergence of *Plasmodium falciparum* resistance to artemisinin and its derivatives has manifested in the form of delayed parasite clearance following treatment with artesunate monotherapy or ACT (1, 2). In areas where artemisinin resistance is emerging, the partner drugs within the combination are under increasing pressure for the selection of resistance. In this context, the identification of molecular markers of resistance to these partner drugs is urgently needed for monitoring the emergence and spread of antimalarial drug resistance.

The ubiquitin system is one of the principal pathways used by all eukaryotic cells to regulate protein abundance levels and protein activities. The chemical modification of proteins by ubiquitin, known as ubiquitylation, is an extremely important posttranslational event that is crucial to numerous cellular processes. Ubiquitin is a conserved 76-amino-acid polypeptide that is covalently attached to one or more lysine residues on proteins through a complex enzymatic cascade. This multienzyme complex includes an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin protein ligase (3). Ubiquitylation begins with the activation of ubiquitin via the E1 ubiquitin-activating enzyme. Subsequently, the E2 ubiquitin-conjugating enzyme catalyzes the transfer of ubiquitin from the E1 enzyme to a conserved cysteine on the E2 enzyme by a transesterification reaction. The final step in the ubiquitylation process is the targeted attachment of ubiquitin to a protein via the E3 ubiquitin ligase, creating an isopeptide bond between a lysine on the target protein and the C-terminal glycine on ubiquitin. E3 proteins are categorized into three major classes of enzymes, homologous to E6-associated protein C terminus (HECT) ligases, really interesting new gene (RING) fingers, and U-box E3 ligases, which are the main determinants of substrate specificity. The ubiquitin system could be a target for antimalarial drugs and involved in resistance (4, 5). Using a genome-wide association study (GWAS), mutations in the E2 ubiquitin-conjugating enzyme gene (PF3D7_1243700) and the HECT E3 ubiquitin ligase gene (PF3D7_0826100) were found to be associated with resistance to pyrimethamine in 45 Senegalese isolates (6). The HECT E3 ubiquitin ligase gene may also be involved in reduced susceptibility to quinine and quinidine (7). Another E3 ubiquitin protein ligase (PF3D7_0627300) belonging to the RING-type E3 ligase is crucial for facilitating the transfer of ubiquitin from the E2 enzyme to a primary amino group of the substrate. It was previously shown that *P. falciparum* parasites carrying the mutant D113N allele for this gene were less susceptible *in vitro* to chloroquine and amodiaquine (8).

The objective of the current study was to identify polymorphisms in the E3 ubiquitin protein ligase gene (PF3D7_0627300) in *P. falciparum* Senegalese isolates and to then evaluate the association of polymorphisms with *ex vivo* susceptibility to chloroquine (CQ), quinine (QN), monodesethylamodiaquine (DQ),

**Received** 27 December 2015 **Returned for modification** 6 March 2016
**Accepted** 8 May 2016

**Accepted manuscript posted online** 16 May 2016

**Citation** Gendrot M, Fall B, Madamet M, Fall M, Wade KA, Amalvict R, Nakoulima A, Benoit N, Diawara S, Diémé Y, Diatta B, Wade B, Pradines B. 2016. Absence of association between polymorphisms in the RING E3 ubiquitin protein ligase gene and *ex vivo* susceptibility to conventional antimalarial drugs in *Plasmodium falciparum* isolates from Dakar, Senegal. Antimicrob Agents Chemother 60:5010–5013. doi:10.1128/AAC.03105-15.

Address correspondence to Bruno Pradines, bruno.pradines@free.fr.

mefloquine (MQ), lumefantrine (LMF), piperaquine (PPQ), pyronaridine (PND), dihydroartemisinin (DHA), artesunate (AS), and doxycycline (DOX).

Forty *P. falciparum* isolates from falciparum malaria patients attending the Hôpital Principal de Dakar from November 2013 to January 2014 and August 2014 to December 2014 were successfully evaluated. Seventy-five percent of the patients were recruited from the emergency department. The other patients were recruited from the intensive care unit (10%), pediatric department (7.5%), and other units (7.5%). There was no information available on antimalarial treatment prior to admission. Despite the WHO's recommendations, the patients were treated with quinine until November 2014 and with artesunate or artemether-lumefantrine at the Hôpital Principal de Dakar. Informed verbal consent was obtained from the patients or their parents/guardians before blood collection. The study was approved by the ethical committee of the Hôpital Principal de Dakar.

Venous blood samples were collected in Vacutainer ACD tubes prior to patient treatment. A malaria diagnosis was confirmed using a thin blood smear and a rapid diagnosis test. Thin blood smears were stained using a RAL kit (Réactifs RAL, Paris, France) based on eosin and methylene blue and were examined to determine *P. falciparum* density and to confirm species monoinfection. The parasitemia was expressed by the number of parasite-infected red cells as a percentage of the total number of red blood cells. The level of parasitemia ranged from 0.06% to 14.1%.

The susceptibility of the isolates was assessed without culture adaptation. For the *ex vivo* microtest, 100 μl of parasitized red blood cells (final parasitemia, 0.5%; final hematocrit, 1.5%) was divided into aliquots and placed into 96-well plates predosed with antimalarial drugs (CQ, QN, MQ, DQ, LMF, DHA, AS, PPQ, PND, and DOX). The plates were incubated in a sealed bag for 72 h at 37°C with atmospheric generators for capnophilic bacteria using Genbag CO2 at 5% $CO_2$ and 15% $O_2$ (bioMérieux, Marcy l'Etoile, France). The *ex vivo* HRP2 enzyme-linked immunosorbent assay (ELISA)-based procedure performed using a commercial Malaria Ag CELISA kit (reference KM2159; Cellabs PTY Ltd., Brookvale, Australia) was previously described (9). The batches of plates were tested and validated with the chloroquine-resistant W2 strain (Indochina) (MR4, Virginia, USA) in three to six independent experiments under the same conditions.

E3 ubiquitin protein ligase (PF3D7_0627300) was amplified by PCR using the following primer pair: 5′-AAT-GGT-CCA-GAA-GAA-GAT-TAT-3′ and 5′-AAA-TAT-ATA-AGG-ATA-GGA-AG-3′. The reaction mixture consisted of 200 ng of genomic DNA, 0.32 μM (each) primers, 1× reaction buffer [750 mM Tris-HCl, 200 mM $(NH_4)_2SO_4$, 0.1% (vol/vol) Tween 20, and stabilizer, pH 8.8], 2.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate (dNTP) mixture, and 0.2 U of Hot Diamond *Taq* polymerase (Eurogentec) in a final volume of 25 μl. The thermal cycler (T3 Biometra) was programmed as follows: 10 min at 95°C followed by 40 cycles of 30 s at 95°C, 45 s at 45°C, and 90 s at 72°C and a final extension of 10 min at 72°C. The purified amplicons were sequenced using the PCR primers and a sequencing primer (5′-AAT-ACT-TAT-GAT-ATG-ACA-AGT-GA-3′) on an ABI Prism 3100 Analyzer (Applied Biosystems) according to the manufacturer's instructions. The sequences were analyzed using Vector NTI Advance software (version 11; Invitrogen, Cergy Pontoise, France).

The 50% inhibitory concentrations ($IC_{50}$s) in the *ex vivo* chemosusceptibility assay ranged from 6.3 to 414.0 nM for CQ, 6.2 to 1,429.8 nM for QN, 1.9 to 227.3 nM for DQ, 0.6 to 45.0 nM for LMF, 3.4 to 74.5 nM for MQ, 3.9 to 241.9 nM for PPQ, 0.4 to 111.6 nM for PND, 0.1 to 17.3 nM for DHA, 0.1 to 18.1 nM for AS, and 0.9 to 79.0 μM for DOX. The only polymorphism identified in the E3 ubiquitin protein ligase gene was the D113N mutation. The sequences of wild-type parasites (Sub 1478173) and mutant parasites (Sub 1478169) were submitted to GenBank. The mutation was present in 25 isolates (62.5% of the 40 samples). However, the D113N mutation was not significantly associated with the $IC_{50}$ of CQ, QN, DQ, MQ, LMF, PPQ, PND, DHA, AS, and DOX (*P* value between 0.2872 and 0.9883 [Wilcoxon signed-rank test]) (Table 1). We did not find in field isolates that *P. falciparum* parasites carrying the mutation D113N allele for this gene were less susceptible *in vitro* to CQ and DQ as previously shown in genetically modified parasites (8). The isolates were categorized as being susceptible or resistant or showing reduced susceptibility using the following cutoff values: 77 nM (CQ), 61 nM (DQ), 115 nM (LMF), 12 nM (DHA and AS), 611 nM (QN), 30 nM (MQ), 135 nM (PPQ), 60 nM (PND), and 37 μM (DOX) (10, 11). These cutoff values for reduced *in vitro* susceptibility to antimalarial drugs were previously estimated using the arithmetic mean plus two standard deviations of the $IC_{50}$s (10, 11). There was no significant difference between the wild-type parasites (wild-type D113N) and the mutated parasites (mutated D113N) in reduced susceptibility to the 10 conventional antimalarial drugs (*P* value between 0.0938 and 1 [chi-square test]) (Table 2). The most notable difference seen was for PPQ, for which 0% of the wild-type isolates had reduced susceptibility versus 19% of the samples with D113N-mutated parasites (*P* value = 0.0938). In 2006, the first-line therapy for uncomplicated malaria became artemether-lumefantrine or artesunate-amodiaquine. The dihydroartemisinin-piperaquine combination was then recommended as a second-line treatment for uncomplicated *P. falciparum* malaria in Senegal. But it seems that the combination dihydroartemisinin-piperaquine is not yet widely used in Da-

**TABLE 1** *Ex vivo* susceptibility of 40 *Plasmodium falciparum* isolates to chloroquine, quinine, monodesethylamodiaquine, mefloquine, lumefantrine, piperaquine, pyronaridine, dihydroartemisinin, artesunate, and doxycycline according to the D113N mutation in the E3 ubiquitin protein ligase gene[a]

| | Wild-type D113N (no. of isolates) | | Mutated D113N (no. of isolates) | | |
|---|---|---|---|---|---|
| Drug | Mean $IC_{50}$ | 95% CI | Mean $IC_{50}$ | 95% CI | *P* value |
| CQ | 75.8 nM (15) | 43.9–130.8 | 60.5 nM (25) | 40.4–89.3 | 0.3909 |
| QN | 116.2 nM (15) | 64.6–209.0 | 71.4 nM (24) | 42.0–121.2 | 0.4188 |
| DQ | 23.5 nM (15) | 12.0–46.2 | 17.1 nM (25) | 10.2–28.7 | 0.4178 |
| MQ | 22.4 nM (14) | 15.3–32.8 | 27.5 nM (25) | 19.0–39.8 | 0.2887 |
| LMF | 6.6 nM (15) | 3.3–13.6 | 5.7 nM (25) | 3.3–9.8 | 0.6711 |
| PPQ | 34.5 nM (13) | 27.1–43.9 | 37.7 nM (25) | 23.0–61.9 | 0.5827 |
| PND | 10.3 nM (13) | 7.6–13.9 | 9.7 nM (20) | 5.8–16.2 | 0.6717 |
| DHA | 1.6 nM (14) | 0.9–2.7 | 1.4 nM (25) | 0.7–2.6 | 0.9883 |
| AS | 3.3 nM (13) | 1.9–5.7 | 2.2 nM (19) | 1.2–4.1 | 0.2872 |
| DOX | 12.2 μM (14) | 6.7–22.3 | 13.6 μM (25) | 8.4–22.1 | 0.6539 |

[a] CQ, chloroquine; QN, quinine; DQ, monodesethylamodiaquine; MQ, mefloquine; LMF, lumefantrine; PPQ, piperaquine; PND, pyronaridine; DHA, dihydroartemisinin; AS, artesunate; DOX, doxycycline; mean $IC_{50}$, geometric mean 50% inhibitory concentration; 95% CI, 95% confidence interval. *P* values were determined by the Wilcoxon signed-rank test.

TABLE 2 Prevalences of *Plasmodium falciparum* isolates with reduced susceptibility to chloroquine, quinine, monodesethylamodiaquine, mefloquine, lumefantrine, piperaquine, pyronaridine, dihydroartemisinin, artesunate, and doxycycline according to the D113N mutation in the E3 ubiquitin protein ligase gene[a]

| Drug | Reduced-susceptibility cutoff | % of isolates with reduced susceptibility (no. of isolates with reduced susceptibility/total no. of isolates) | | *P* value |
|---|---|---|---|---|
| | | Wild-type group | Mutated group | |
| CQ | 77 nM | 60.0 (9/15) | 52.0 (13/25) | 0.6224 |
| QN | 611 nM | 6.7 (1/15) | 4.2 (1/24) | 0.7305 |
| DQ | 61 nM | 33.3 (5/15) | 16.0 (4/25) | 0.2037 |
| MQ | 30 nM | 42.9 (6/14) | 52.0 (13/25) | 0.5807 |
| LMF | 115 nM | 0 (0/15) | 0 (0/25) | 1.0000 |
| PPQ | 135 nM | 0 (0/13) | 20.0 (5/21) | 0.0938 |
| PND | 60 nM | 0 (0/13) | 10.0 (2/20) | 0.2394 |
| DHA | 12 nM | 0 (0/14) | 4.0 (1/25) | 0.4423 |
| AS | 12 nM | 7.7 (1/13) | 10.5 (2/19) | 0.7870 |
| DOX | 37 μM | 14.3 (2/14) | 28.0 (7/25) | 0.3295 |

[a] CQ, chloroquine; QN, quinine; DQ, monodesethylamodiaquine; MQ, mefloquine; LMF, lumefantrine; PPQ, piperaquine; PND, pyronaridine; DHA, dihydroartemisinin; AS, artesunate; DOX, doxycycline. *P* values were determined using the chi-square test.

kar. Multidrug resistance to dihydroartemisinin-piperaquine has emerged in Cambodia, where the rate of recrudescent infections in patients increased from 15.4% in 2011 to 2013 to 39% in 2012 to 2014 (12, 13). In 2012 to 2014, 57% of the patients were still parasitemic at 72 h (13). Based on data from 40 *P. falciparum* isolates, there was no association between the D113N mutation and *ex vivo* susceptibility to 10 conventional antimalarial drugs, although more data may show a different outcome.

The main limitation of the current study was the low number of *P. falciparum* isolates. It is difficult to extrapolate the results to other parts of the world without a new set of data. Another limitation was the use of the standard *in vitro* test for artemisinin exploration of resistance. The standard *in vitro* test was not adapted to follow resistance to artemisinin derivatives. The clinical resistance to artemisinin was manifested by an increase in the ring-stage survival rate after contact with artemisinin (ring survival test) (14). The ubiquitin system remains a target which may be involved in reduced susceptibility to antimalarial drugs. Furthermore, more isolates from different geographical areas, and, more specifically, from Cambodia, where multidrug resistance to dihydroartemisinin-piperaquine has emerged, are needed to ascertain the role of the different enzymes in this system in *P. falciparum* resistance.

## ACKNOWLEDGMENTS

We thank the patients and the staff of the Hôpital Principal de Dakar and Ndeye Fatou Diop and Maurice Gomis from the Hôpital Principal de Dakar for technical support.

We declare that we have no conflicting interests.

This research was supported by the Délégation Générale pour l'Armement (grant no. PDH-2-NRBC-4-B1-402), by the Schéma directeur Paludisme, Etat Major des Armées Françaises (grant LR 607a), and by the Ministère des Affaires Etrangères.

## FUNDING INFORMATION

This work, including the efforts of Bruno Pradines, was funded by Délégation Générale pour l'Armement (PDH-2-NRBC-4-B1-402). This work, including the efforts of Bruno Pradines, was funded by Etat Major des Armées Françaises (LR 607A). This work, including the efforts of Bruno Pradines, was funded by Ministère des Affaires Etrangères.

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

## REFERENCES

1. **Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwrong M, Chotivanish K, Lim P, Herdman T, An SS, Yeung S, Singhasivanon P, Day NPJ, Lindegardh N, Socheat D, White NJ.** 2009. Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med **361:**455–467. http://dx.doi.org/10.1056/NEJMoa0808859.
2. **Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, Seng S, Anderson JM, Mao S, Sam B, Sopha C, Chuor CM, Nguon C, Sovannaroth S, Pukrittayakamee S, Jttamala P, Chotivanich K, Chutasmit K, Suchatsoonthorn C, Runcharoen R, Hien TT, Thuy-Nhien NT, Thanh NV, Phu NH, Htut Y, Han KT, Aye KH, Mokuolu OA, Olaosebikan RR, Folaranmi OO, Mayxay M, Khanthavong M, Hongvanthong B, Newton PN, Onyamboko MA, Fanello CI, Tshefu AK, Mishra N, Valecha N, Phyo AP, Nosten F, Yi P, Tripura R, Borrmann S, Bashraheil M, Peshu J, Faiz MA, Ghose A, Hossain MA, Samad R, et al.** 2014. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med **371:**411–423. http://dx.doi.org/10.1056/NEJMoa1314981.
3. **Hershko A, Ciechanover A.** 1998. The ubiquitin system. Annu Rev Biochem **67:**425–479. http://dx.doi.org/10.1146/annurev.biochem.67.1.425.
4. **Hamilton MJ, Lee M, Le Roch KG.** 2014. The ubiquitin system: an essential component to unlocking the secrets of malaria parasite biology. Mol Biosyst **10:**715–723. http://dx.doi.org/10.1039/c3mb70506d.
5. **Volkman SK, Neafsey DE, Schaffner SF, Park DJ, Wirth DF.** 2012. Harnessing genomics and genome biology to understand malaria biology. Nat Rev Genet **13:**315–328. http://dx.doi.org/10.1038/nrg3187.
6. **Park DJ, Lukens AK, Neafsey DE, Schaffner SF, Chang HH, Valim C, Ribacke U, Van Tyne D, Galinsky K, Galligan M, Becker JS, Ndiaye D, Mboup S, Wiegand RC, Hartl DL, Sabeti PC, Wirth DF, Volkman SK.** 2012. Sequence-based association and selection scans identify drug resistance loci in the *Plasmodium falciparum* malaria parasite. Proc Natl Acad Sci U S A **109:**13052–13057. http://dx.doi.org/10.1073/pnas.1210585109.
7. **Sanchez CP, Liu CH, Mayer S, Nurhasanah A, Cyrklaff M, Mu J, Ferdig MT, Stein WD, Lanzer M.** 2014. A HECT ubiquitin-protein ligase as a novel candidate gene for altered quinine and quinidine responses in *Plasmodium falciparum*. PLoS Genet **10:**e1004382. http://dx.doi.org/10.1371/journal.pgen.1004382.
8. **Ribacke U, Martlett M, Patel SD, Seneratne N, Park DJ, Duraisingh M, Sabeti PC, Volkman SK, Wirth DF.** 2012. Adaptative evolution of a ring ubiquitin ligase mediates reduced drug sensitivity in *Plasmodium falciparum*, p 299. Abstr 61st Meet Am Soc Trop Med Hyg, Atlanta, GA, 11–15 November 2012.
9. **Fall B, Camara C, Fall M, Nakoulima A, Dionne P, Diatta B, Diemé Y, Wade B, Pradines B.** 2015. *Plasmodium falciparum* susceptibility to standard and potential anti-malarial drugs in Dakar, Senegal, during the 2013–2014 malaria season. Malar J **14:**60. http://dx.doi.org/10.1186/s12936-015-0589-3.
10. **Fall B, Diawara S, Sow K, Baret E, Diatta B, Fall KB, Mbaye PS, Fall F, Diémé Y, Rogier C, Wade B, Bercion R, Pradines B.** 2011. *Ex vivo* susceptibility of *Plasmodium isolates* from Dakar, Senegal, to seven standard anti-malarial drugs. Malar J **10:**310. http://dx.doi.org/10.1186/1475-2875-10-310.
11. **Pascual A, Madamet M, Briolant S, Gaillard T, Amalvict R, Benoit N, Travers D, Pradines B.** 2015. Multinormal *in vitro* distribution of *Plasmodium falciparum* susceptibility to piperaquine and pyronaridine. Malar J **14:**49. http://dx.doi.org/10.1186/s12936-015-0586-6.
12. **Leang R, Taylor WRJ, Mey Bouth D, Song L, Tarning J, Chuor Char M, Kim S, Witkowski B, Duru V, Domergue A, Khim N, Ringwald P,**

**Ménard D.** 2015. Evidence of *Plasmodium falciparum* malaria multidrug resistance to artemisinin and piperaquine in Western Cambodia: dihydroartemisinin-piperaquine open-label multicentre clinical assessment. Antimicrob Agents Chemother **59:**4719–4726. http://dx.doi.org/10.1128/AAC.00835-15.

13. **Spring M, Lin JT, Manning JE, Vanachayangkul P, Somethy S, Bun R, Se Y, Chann S, Ittiverakul M, Sia-Ngam P, Kuntawunginn W, Arsanok M, Buathong N, Chaorattanakawee S, Gosi P, Ta-Aksorn W, Chanarat N, Sundrakes S, Kong N, Kheang Heng T, Nou S, Teja-Isavadharm P, Pichyangkul S, Thang Phann S, Balasubramanian S, Juliano JJ, Meshnick SR, Meng Chour C, Prom S, Lanteri CA, Lon C, Saunders DL.** 2015. Dihydroartemisinin-piperaquine failure associated with a triple mutant including Kelch13 C580Y in Cambodia: an observational cohort study. Lancet Infect Dis **15:**683–693. http://dx.doi.org/10.1016/S1473-3099(15)70049-6.
14. **Witkowski B, Amaratunga C, Khim N, Sreng S, Chim P, Kim S, Lim P, Mao S, Sopha C, Sam B, Anderson JM, Duong S, Chuor CM, Taylor WR, Suon S, Mercereau-Puijalon O, Fairhurst RM, Menard D.** 2013. Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: in-vitro and ex-vivo drug-response studies. Lancet Infect Dis **13:**1043–1049. http://dx.doi.org/10.1016/S1473-3099(13)70252-4.