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Susceptibility of human *Plasmodium knowlesi* infections to anti-malarials

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Abstract

Background: Evidence suggests that *Plasmodium knowlesi* malaria in Sarawak, Malaysian Borneo remains zoonotic, meaning anti-malarial drug resistance is unlikely to have developed in the absence of drug selection pressure. Therefore, adequate response to available anti-malarial treatments is assumed.

Methods: Here the *ex vivo* sensitivity of human *P. knowlesi* isolates in Malaysian Borneo were studied, using a WHO schizont maturation assay modified to accommodate the quotidian life cycle of this parasite. The *in vitro* sensitivities of *P. knowlesi* H strain adapted from a primate infection to *in vitro* culture (by measuring the production of *Plasmodium* lactate dehydrogenase) were also examined together with some assays using *Plasmodium falciparum* and *Plasmodium vivax*.

Results: *Plasmodium knowlesi* is uniformly highly sensitive to artemisinins, variably and moderately sensitive to chloroquine, and less sensitive to mefloquine.

Conclusions: Taken together with reports of clinical failures when *P. knowlesi* is treated with mefloquine, the data suggest that caution is required if using mefloquine in prevention or treatment of *P. knowlesi* infections, until further studies are undertaken.

Keywords: Artemisinin, Artemether, Artesunate, Dihydroartemisinin, DHA, Chloroquine, Mefloquine, Malaria

Background

From its natural simian hosts in Southeast Asia, *Plasmodium knowlesi* has emerged as a significant human pathogen, particularly in Malaysian Borneo [1-3]. Human *P. knowlesi* infections cause febrile illnesses that can rapidly progress to severe and sometimes fatal outcomes [4]. Ominously, the incidence of *P. knowlesi* malaria is increasing in geographic areas where *Plasmodium falciparum* and *Plasmodium vivax* are coming under control, thereby threatening the aim of eliminating malaria [5]. Determining the efficacy of conventional anti-malarials against *P. knowlesi* is a priority, particularly as there are no reports of anti-malarials assessed against human isolates of *P. knowlesi ex vivo*.

Here, the drug sensitivity profiles of *P. knowlesi* isolates obtained from patients being recruited into a study of the pathophysiology of knowlesi malaria in an endemic area of Sarawak, Malaysian Borneo were investigated. Currently, the WHO recommends artemisinin-based combination therapy (ACT) as first-line treatment for malaria in most endemic areas, so artemisinin and its clinically useful derivatives artesunate, dihydroartemisinin (DHA) and artemether were tested. Mefloquine, used as a partner drug in certain artemisinin-based combinations and in prophylaxis against malaria, and chloroquine that is recommended for treatment of *Plasmodium malariae* (the species which *P. knowlesi* is often confused with when diagnosed by microscopy) were also included. In addition, results from *P. falciparum* and *P. vivax* studied contemporaneously are presented, together with those obtained with the H strain laboratory isolate of *P. knowlesi* (cultured *in vitro* in rhesus erythrocytes) to confirm the methodologies used. Insights into the drug susceptibility patterns of this important emerging parasite, may prove useful in guiding

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the best choice of anti-malarial treatment regimens for *P. knowlesi* infection.

Methods

Patient recruitment

Plasmodium isolates were obtained from patients presenting to hospitals in Sarikei and Sibü. Informed written consent was obtained from all patients entered into this study, which was approved by the Malaysian Ministry of Health's Medical Research and Ethics Committee, and the Sarawak State Planning Unit. Infecting species was confirmed by *Plasmodium* species-specific nested-PCR assays [6] and only patients with single species infections were retained in the study.

Blood collection and *ex vivo* parasite development in growth assays

Pre-treatment venous blood from each patient was collected into EDTA. Parasitaemia and the asexual stage of development were determined by Giemsa-stained thin film microscopy. Whole blood (~2.5 ml) was washed twice without centrifugation to avoid haemolysis, before resuspending in RPMI 1640 complete medium supplemented with 20 mM D-glucose, 40 mM HEPES, 25 mg/l gentamicin sulphate, and 15% v/v human AB plasma with 0.2 mM hypoxanthine.

Growth inhibition by anti-malarials was assessed by quantifying schizont maturation using an adapted WHO Mark III assay [7]. Species of *Plasmodium* tested and the time lag in maturation seen previously *ex vivo* were allowed for in these assays [8]. While more complex counting procedures have been used to study *P. vivax* parasites that, like *P. knowlesi*, have mature parasites present in circulating blood [9], only the timing of assays was altered. This is because the *P. knowlesi* isolates contained predominantly immature parasites (Table 1) and results from a parallel study on tightly synchronised

immature laboratory *P. knowlesi* H strain parasites with artesunate using the *pLDH* (see below) and Mark III assays were comparable with each other and with the data derived from isolates (see Results).

Aliquots (100 µl) of no drug control and serial dilutions of the anti-malarial compounds in culture medium (final concentrations of 0.25 to 25 nM for artemisinin and derivatives and 1.25 nM to 1 µM for chloroquine and mefloquine) were dispensed into 96-well plates, and parasites were added (100 µl at 2% haematocrit). Incubation was in 5% O₂, 5% CO₂, 90% N₂ at 37°C until the majority of parasites reached the schizont stage (with at least 3 nuclei after 12–17 h for *P. knowlesi*). Monitoring of maturation was undertaken every 2 h by examination of fixed, Giemsa stained thin blood films, taken from a parallel culture to those of the drug exposed cultures. When at least half of the parasites in the monitoring culture had reached the schizont stage of development, the drug exposed cultures and their controls were harvested, as thick films on glass slides, and examined. The duration of *in vitro* development for each isolate (as determined above), which also equates to the time of drug exposure, is given in Table 1. Thick films were fixed by air drying for at least 24 h, stained with Giemsa, and mounted to protect slides during transportation.

As thick films are easily damaged, five replicates were prepared for each drug concentration and 3 replicates then counted for each experimental condition. Counting was in a blinded fashion to avoid bias. Thick films were counted according to the WHO Mark III protocol. At least 200 asexual parasites were counted. Fields of view were consecutive, starting at the left edge of each blood film and moving stepwise in a uniform direction (to ensure no overlap). Asexual parasites were grouped into either trophozoites or schizonts (defined as asexual parasites displaying 1–2 nuclei and 3 or more nuclei, respectively).

Table 1 Patient isolate data

Isolate	Species	% starting parasitaemia	% schizont at start of assay	Duration of <i>in vitro</i> development (h)	% schizont at end of assay
P0002	<i>Pk</i>	0.4	15	12	80
P0003	<i>Pk</i>	0.6	10	12	86
P0006	<i>Pk</i>	0.8	33	17	53
P0009	<i>Pk</i>	7.0 ^a	17	15	94
P0010	<i>Pk</i>	0.8	6	12	63
P0011	<i>Pk</i>	1.3	8	14	54
P0007	<i>Pf</i>	2.0 ^a	0	31	50
P0001	<i>Pv</i>	0.5	20	18	58
P0013	<i>Pv</i>	0.5	30	29	60

^aAbove Mark III assay cut-off (for examination of *P. falciparum* mono-infections), assuming a parasitaemia of 1.6% equates to approximately 80,000 parasites per µl of blood, and was used undiluted. Note that in both cases good schizont development was observed. For each isolate, the species, parasitaemia and change in development over time are presented.

Growth assay measuring *Plasmodium lactate dehydrogenase* (pLDH)

Rhesus monkey red blood cells for *in vitro* *P. knowlesi* culture were obtained under protocols approved by the independent institutional ethical committee (DEC) according to Dutch and European laws.

The efficacy of the anti-malarial compounds was also assessed *in vitro* against the laboratory maintained *P. knowlesi* H strain [10,11], by measuring the production of pLDH [12]. pLDH catalyzes the conversion of 3-acetylpyridine adenine dinucleotide (APAD) and lactate to APADH and pyruvate. The enzyme diaphorase subsequently converts nitro blue tetrazolium (NBT) to nitro blue formazan (NBF), using APADH as a reducing agent. NBF can be measured at a wavelength of 655 nm. Serial dilutions of the anti-malarial compounds (100 μ l in culture medium) and including a no drug control were dispensed into 96-well plates, to which tightly synchronized (by alanine lysis) early ring-stage infected erythrocytes (100 μ l at 2% haematocrit and 2% parasitaemia in culture medium) were added. The plates were then placed in 3% O₂, 7% CO₂, 90% N₂ at 37°C for 22 h to mature (just prior to parasite release). Growth was halted and drug removed, by washing twice in ice-cold PBS. Erythrocytes were lysed by freezing at -20°C and thawing. Aliquots of 0.5 mg/ml of NBT, 1 U/ml diaphorase and 50 μ g/ml APAD in LDH buffer (100 mM Tris-HCl, pH 8.0, 50 mM Na L-lactate, 2.5% v/v Triton X-100) were added to the thawed cell pellets and incubated for 30 min in the dark at room temperature with shaking. The optical density (OD) at 655 nm of each well was measured in a BioRad 680 microplate reader. OD₆₅₅ values were used to calculate growth by comparing the OD₆₅₅ values at each drug concentration with that of the no drug control. To test the efficacy of artemisinin, artemether, artesunate and DHA, preparations of final concentrations of 0.1 nM to 25 nM were used, and for chloroquine and mefloquine, final concentrations of 1.25 nM to 1 μ M were used.

IC₅₀ values and sequence alignments

Dose-response data were modeled using a four-parameter fit and a variable slope, using Prism (Version 4.0a). Goodness of fit was assessed by R², and either noted in the text or highlighted if < 0.8 in tables and figures. For the schizont maturation assay, the top parameter was constrained to 100% for assays using artemisinins but not for mefloquine or chloroquine. For the pLDH assay, the top and bottom parameters were constrained to 100 and 0, respectively, for all drugs and, for the ³H-hypoxanthine incorporation assay, the top parameter was constrained to 100. Data are summarized as a mean and 95% CI for replicates of single parasite assays and a mean \pm SEM for results from multiple independent parasite assays.

The *P. knowlesi* and *P. vivax* orthologues of *P. falciparum* CRT, MDR1, and ATP6 (proteins associated with modulating sensitivities to chloroquine, mefloquine and artemisinins [13]), were aligned with *P. falciparum* 3D7 strain, as a drug sensitive control. H strain and Sal-1 strain sequences were used for *P. knowlesi* and *P. vivax*, respectively. Wild-type and polymorphism sequence data were taken from PlasmoDB [14]. Alignments were performed in MacVector (version 11.0.2).

Results

Patient recruitment

Patient isolate data from those obtained in Sarikei and Sibuhut hospitals, between March and September 2010, are shown in Table 1. A total of nine patients were recruited into this study, six with *P. knowlesi*, two with *P. vivax* and one with *P. falciparum* mono-infections. Infections were initially diagnosed by microscopy and confirmed later by nested PCR [2,6].

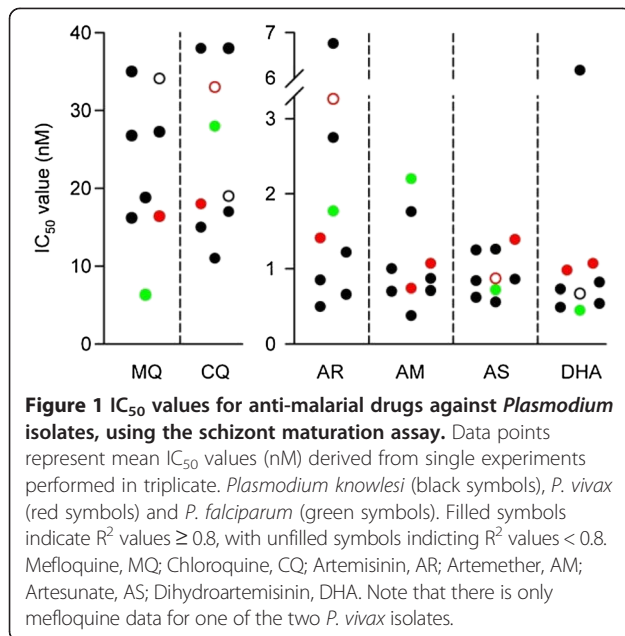
Parasite development

In vitro development data for each isolate used during growth assays are shown in Table 1. *P. knowlesi* field isolates were seeded into growth assays with a starting parasitaemia ranging from 0.4 to 7%. The percentage of schizonts present at the start of the assays ranged from 6 to 33%. Good *in vitro* development was demonstrated for all *P. knowlesi* field isolates, with 53 to 94% of asexual parasites reaching the schizont stage, over a development period of 12 to 17 h. Development was similar for *P. falciparum* and *P. vivax* field isolates (Table 1).

Growth assays: schizont maturation

Dose-response curves were used to derive IC₅₀ values, which are presented in Figure 1, together with mean values in Table 2. All the isolates from humans were sensitive to artemisinin and its derivatives, with IC₅₀ values in the low nM range (from 0.38 to 6.8 nM). The IC₅₀ values for chloroquine on *P. knowlesi* from humans were relatively high, with values ranging from 11 to 38 nM but were similar to those derived for the single *P. falciparum* isolate (28 (20 to 38) nM; R² = 0.93) and the two *P. vivax* isolates (33 (10 to 110) and 18 (11 to 27) nM; R² = 0.45 and 0.95, respectively) that were studied.

Plasmodium knowlesi isolates were least sensitive to mefloquine, with a mean IC₅₀ value of 26 nM (Table 2). The IC₅₀ value for mefloquine on the single *P. falciparum* field isolate (6.3 (2.5 to 16) nM; R² = 0.97) was four-fold less than that of the average *P. knowlesi* IC₅₀ value and 2.5-fold less than the lowest *P. knowlesi* IC₅₀ value (16 (12 to 22) nM; R² = 0.96).



Growth assays: pLDH

To validate the results of the drug assays on *P. knowlesi* from patients, anti-malarials were assessed against the well characterized *P. knowlesi* H strain grown *in vitro* in rhesus erythrocytes. To determine whether data generated by the pLDH method (as used to study the H strain) would be comparable with the adapted schizont maturation method (as used in the field study) a parallel study was performed, using artesunate. The IC_{50} value for artesunate against the H strain was 2.0 (0.93 to 4.2) nM ($R^2 = 0.39$), using the pLDH assay (from a single experiment repeated in quintuplicate), and 1.2 (0.88 to 1.6) nM ($R^2 = 0.95$), using the schizont maturation assay (from a single experiment repeated in triplicate), demonstrating that the two assay methods are comparable, at least in the

Table 2 *P. knowlesi* drug sensitivity data

Drug	IC_{50} values (nM) for <i>P. knowlesi</i> isolates (maturation assay) ^a	IC_{50} values (nM) for <i>P. knowlesi</i> H strain (pLDH assay) ^b
Mefloquine	26 (± 3.1)	25 (7.4 to 81) ^c
Chloroquine	23 (± 4.8)	3.2 (2.2 to 4.7)
Artemisinin	2.1 (± 0.99)	0.80 (0.35 to 1.9) ^c
Artemether	0.90 (± 0.19)	0.84 (0.34 to 2.1) ^c
Artesunate	0.90 (± 0.12)	2.0 (0.93 to 4.2) ^c
DHA	1.6 (± 0.92)	0.79 (0.62 to 1.0)

^aMean (\pm SEM), from 6 independent experiments (each of which was performed in triplicate).

^bMean (95% CIs), from a single experiment repeated in quintuplicate.

^c $R^2 < 0.8$.

IC_{50} values are presented for six anti-malarials on *P. knowlesi* field isolates and laboratory H strain.

case of artemisinins. Further IC_{50} values derived from pLDH assays are presented in Table 2. All IC_{50} values for field isolates and the *P. knowlesi* H strain for artemisinins are highly comparable.

For mefloquine, the IC_{50} value against the laboratory H strain (25 (7.4 to 81) nM; $R^2 = 0.40$) was nearly identical to the mean IC_{50} value of human isolates (26 nM). The IC_{50} values suggest that *P. knowlesi* may be intrinsically insensitive to mefloquine. To ensure that the mefloquine stock used in field studies had not degraded during the study, an aliquot was tested after shipping it back to St. George's, University of London after the study. Using hypoxanthine incorporation as a measure of growth [15] in a single assay (performed in quadruplicate) with *P. falciparum* (3D7) parasites cultured *in vitro* in human erythrocytes, the IC_{50} value was 9.7 (5.5 to 17) nM ($R^2 = 0.89$), confirming that potency of mefloquine was maintained.

Interestingly, the H strain was over 7-fold more sensitive to chloroquine than the average value for field isolates, having an IC_{50} value of 3.2 (2.2 to 4.7) nM ($R^2 = 0.86$). This value was also well below the lowest calculated IC_{50} value (11 (5.3 to 24) nM; $R^2 = 0.94$) for a field isolate.

Sequence alignments

Point mutations associated with change in the sensitivity of *P. falciparum* to chloroquine, mefloquine and the artemisinins [13] were analysed, and similarities and differences in sequences encoded by *P. knowlesi* and *P. vivax* homologues are reported in Figure 2. Known loci at which mutations can reduce drug sensitivity were highly conserved between *P. knowlesi* H, *P. vivax* Sal-1 MDR1 homologues and MDR1 in drug sensitive *P. falciparum* 3D7. PfATP6 orthologues demonstrated polymorphism in amino acids in about two thirds of the

PfCRT			PfATP6			PfMDR1		
Pf	Pk	Pv	Pf	Pk	Pv	Pf	Pk	Pv
C72S	71C	71C	I89T	89V	89V	N86Y	91N	91N
M74I	73M	73M	H243Y	243H	243H	Y184F	189Y	189Y
N75E	74E	74E	L263E	263S	263A	N1042D	1081N	1082N
K76T	75K	75K	L402V	402A	402A	D1246Y	1293D	1292D
H97Q	96H	96H	E431K	429T	429A			
S219A	218A	218A	E432K	430S	430S			
A220S	219S	219S	Q574P	-	-			
Q271E	270Q	270Q	A623E	-	-			
N326S/D	325N	325N	S769N	708K	718K			
I356T/L	355I	355I						
R371I	370M	370M						

Figure 2 Alignment of CRT, ATP6, and MDR1 homologues of *P. knowlesi* H strain and *P. vivax* Sal-1 strain, against *P. falciparum* 3D7 and strains with known point mutations associated with drug resistance. Amino acids are colour coded, with green representing the drug sensitive *P. falciparum* 3D7, and red representing amino acid changes found in *P. falciparum* isolates that have a change in drug sensitivity.

residues previously examined for effects on drug sensitivity, including L263 [16-18].

The CRT orthologues were fairly well conserved with the exceptions of S219A, A220S, and R371M in the chloroquine sensitive strain. These differences do not confer chloroquine resistance to the *P. knowlesi* H strain. Agreement in sequences in these polymorphic regions was higher between *P. knowlesi* H and *P. vivax* Sal-1 than between either strain and *P. falciparum* 3D7 consistent with a closer phylogenetic relationship between the former species [19].

Discussion

In vitro culture of *P. knowlesi* has only recently been achieved in human erythrocytes [20,21]. However, drug sensitivity assays of natural human infections can only be assessed in short term cultures. These allowed the successful application of drug sensitivity micro-assays such as the WHO Mark III micro-assay test after adaptation to the quotidian life cycle of the parasite and frequent (every 2 to 2.5 h) monitoring of development to allow assessment of when most parasites were mature schizonts in control samples.

The excellent *in vitro* efficacy of the artemisinins against both human *P. knowlesi* (mean IC₅₀ values < 2.2 nM) and the laboratory (H) strain is consistent with recent observations made on patients treated with artesunate, where no mortality was observed after treatment [22]. These results also agree with data from animal models, which demonstrate the successful clearance of *P. knowlesi* parasites from infected rhesus monkeys, by artemisinin in combination therapy with naphthoquine [23]. In addition, William *et al.* [24] in a retrospective analysis of clinical cases of knowlesi malaria in Sabah, reported the successful treatment of patients by artemether-lumefantrine combination therapy. This study also noted that where knowlesi malaria developed signs of severity, intravenous artesunate was effective [24].

Intriguingly, chloroquine IC₅₀ values from this field study, including those derived against *P. knowlesi* (with values ranging from 11 to 38 nM), were higher than that of the laboratory *P. knowlesi* H strain (3.2 nM), although the reason for this is unclear. In general, the values are comparable with chloroquine-sensitive laboratory strains of *P. falciparum*, having IC₅₀ values ranging from 8 to 15 nM [25-27]. These values all fall below the 100 nM threshold used to define chloroquine resistance [27,28] and there is no evidence for clinical chloroquine resistance reported in *P. knowlesi*. Chloroquine is effective both as a monotherapy and when used in combination with primaquine [1,24,29]. Consistent with these findings, Tyagi *et al.* [30] have recently reported that CRT (and DHFR) sequences from *P. knowlesi* clinical isolates collected in the Andaman and Nicobar Islands, India,

were all found to be wild-type (with close homology to the CRT sequence of chloroquine sensitive *P. falciparum* parasites – see Figure 2 and below). Nevertheless, continued monitoring of chloroquine sensitivity in *P. knowlesi* might be prudent bearing in mind the history of chloroquine resistance development in other malarial species.

Evidence suggests that transmission of *P. knowlesi* to humans in Sarawak remains zoonotic and, thus, ostensibly free from mefloquine drug selection pressure. However, results with mefloquine consistently showed a low sensitivity in *P. knowlesi* field isolates, when compared with that of *P. falciparum*. The mean IC₅₀ value for mefloquine calculated for the 6 *P. knowlesi* isolates is 26 nM, which is just above the value used to define mefloquine resistance in *P. falciparum* (>24 nM) in some reports [31,32] but well below that reported by others (>119 nM) [33]. Importantly, the reduced *P. knowlesi* response in human isolates was also observed in the *P. knowlesi* H experimental line. Given the efficacy of the mefloquine used was confirmed after the end of the study, these results indicate an innate tolerance of *P. knowlesi* to mefloquine. These findings also suggest the strong possibility of treatment failure if mefloquine is used as mono or combination therapy for *P. knowlesi* and is supported by reports of mefloquine treatment failure in rhesus monkeys infected with *P. knowlesi* [34], as well as recent cases of mefloquine treatment failure in humans with knowlesi malaria [35]. On this basis, mefloquine should be used with caution for the treatment of knowlesi malaria, or indeed for prophylaxis against malaria in areas where acquiring knowlesi is a risk until larger studies have been undertaken.

There is reasonable conservation of MDR1 sequences between *P. vivax* Sal-1 and *P. knowlesi* H and the *P. falciparum* 3D7 reference strain. Previous studies on *P. falciparum* found that increased *mdr1* copy number conferred a mefloquine resistant phenotype [36] and risk of treatment failure, although *P. knowlesi mdr1* copy number in the isolates reported in the current study have not been determined. Alignments of the *P. vivax* Sal-1 and *P. knowlesi* H CRT and ATP6 orthologues with the *P. falciparum* 3D7 sequence revealed several polymorphic differences between the *P. vivax* and *P. knowlesi* alignments and that of the *P. falciparum*. These substitutions do not alter sensitivity of *P. knowlesi* to artemisinins and give insights into the possible contributions of these residues to artemisinin sensitivity in *P. falciparum*.

Here, it has been established that it is possible to culture *in vitro* *P. knowlesi* in human erythrocytes in the short term, when taken *ex vivo*. In addition, the successful adaptation of the schizont development assay to determine anti-malarial drug sensitivities of *P. knowlesi* field isolates has been shown. Using this adapted method, this study has demonstrated that chloroquine and artemisinin

based drugs are effective against *P. knowlesi* parasites. Conversely this study has shown poor sensitivity of *P. knowlesi* field isolates and laboratory H strain to mefloquine, suggesting innate reduced sensitivity of the parasite to this important anti-malarial drug.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The study was conceived by SK and designed by HMS, CHMK, BS, JCS and SK. The assays were performed by FAF with support from HMS, AS, MAA, LCW, JCS and EMP. The manuscript was prepared by FAF, HMS, and SK. All authors had the opportunity to read and approve the manuscript.

Acknowledgements

We gratefully acknowledge the staff at Hospital Sarikei especially Mr Wong Ching Toh, Mr Pek Peng Chin, Mdm Siti Syartinah and Mdm Raymand Johan for helping with patient recruitment and Mdm Dayang Shuaisah Awang Mohamad and the staff at the Malaria Research Centre at UNIMAS. Finally, we would like to thank the patients who so kindly agreed to be a part of this study, and without whom this research would not have been possible.

Funding

FAF was funded by the MRC-Doctoral Training Grant G0800110. This study was funded by the Medical Research Council (MRC) UK; Grant number G0801971 and the European Community's Seventh Framework Programme (FP7/2007-2013), EVIMALAR network of Excellence under grant agreement N° 242095 and NANOMAL under grant agreement N° 304948.

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Received: 18 September 2013 Accepted: 12 November 2013

Published: 19 November 2013

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doi:10.1186/1475-2875-12-425

Cite this article as: Fatih et al.: Susceptibility of human *Plasmodium knowlesi* infections to anti-malarials. *Malaria Journal* 2013 **12**:425.

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