Sulfadoxine-pyrimethamine impairs *Plasmodium falciparum* gametocyte infectivity and *Anopheles* mosquito survival

Aminatou Kone[a], **Marga van de Vegte-Bolmer**[b], **Rianne Siebelink-Stoter**[b], **Geert-Jan van Gemert**[b], **Antoine Dara**[a], **Hamidou Niangaly**[a], **Adrian Luty**[b], **Ogobara K. Doumbo**[a], **Robert Sauerwein**[b], and **Abdoulaye A Djimde**[a,*]

[a]Malaria Research and Training Center (MRTC), Department of Epidemiology of Parasitic Diseases, Faculty of Medicine, Pharmacy and Odonto-Stomatology, University of Bamako, B.P. 1805, Point G, Bamako, Mali [b]Medical Parasitology-268, MMB-Nijmegen Center for Molecular Life Sciences, Radboud University Nijmegen Medical Center, PO Box 9101, 6500 HB Nijmegen Netherlands

Abstract

Sulfadoxine-pyrimethamine (SP) is currently the drug of choice for intermittent preventive treatment of *Plasmodium falciparum* both in pregnancy and infancy. A prolonged parasite clearance time conferred by *dhfr* and *dhps* mutations is believed to be responsible for increased gametocyte prevalence in SP treated individuals. However, using a direct feeding assay in Mali, we showed that gametocytes present in peripheral venous blood post-SP treatment had reduced infectivity for *Anopheles gambiae* sensu stricto (ss) mosquitoes. We investigated the potential mechanisms involved in the *dhfr* and *dhps* quintuple mutant NF135 and the single *dhps* 437 mutant NF54. Concentrations of Sulfadoxine (S) and Pyrimethamine (P) equivalent to the serum levels of the respective drugs on day 3 (S = 61 ug/ml, P = 154.7 ng/ml) day 7 (S = 33.8 ug/ml, P = 66.6 ng/ml) and day 14 (S = 14.2 ug/ml, P = 15.7 ng/ml) post-SP treatment were used to study the effect on gametocytogenesis, gametocyte maturation and infectivity to *Anopheles stephensi* mosquitoes fed through an artificial membrane. The drugs readily induced gametocytogenesis in the mutant NF-135 strain but effectively killed the wild-type NF54. However, both drugs impaired gametocyte maturation yielding odd-shaped non-exflagelating mature gametocytes. The concomitant ingestion of both S and P together with gametocytemic blood-meal significantly reduced the prevalence of oocyst positivity as well as oocyst density when compared to controls ($P < 0.001$). In addition, day 3 concentrations of SP decreased mosquito survival by up to 65% ($P < 0.001$). This study demonstrates that SP is deleterious in vitro for gametocyte infectivity as well as mosquito survival.

Keywords

Plasmodium falciparum; Sulfadoxine-pyrimethamine; Gametocyte; Impaired gametocytogenesis; Mosquito; Infectivity

*Corresponding author. Abdoulaye A. Djimde, Malaria Research and Training Center, Department of Epidemiology of Parasitic Diseases, University of Bamako, B.P. 1805, Point G, Bamako, Mali Tel./fax: +223 20 22 81 09. adjimde@icermali.org.

1. Introduction

In most tropical and many sub-tropical developing countries, malaria is a major cause of morbidity, especially among young children and pregnant women, and mortality, principally among infants and toddlers. Increasing resistance of *Anopheles* mosquitoes to insecticides has crippled national and regional Malaria Control Programs based on vector control interventions. Furthermore, the spread of chloroquine and Sulfadoxine-Pyrimethamine (SP)-resistant strains of *Plasmodium falciparum* in Asia, Africa and South America has created a public health concern in the clinical treatment and prevention of malaria in many countries.

Although artemisinin-based combination therapies are now the World Health Organization's (WHO) recommended first line drugs for the management of uncomplicated malaria, there are very few options for preventing malaria during pregnancy in endemic countries. Intermittent preventive treatment of malaria (IPT), which is aimed at protecting at-risk populations from both clinical malaria and the sequelae of asymptomatic parasitemia (Kayentao et al., 2005; Manzi et al., 2008) continues to heavily rely on SP-containing regimens (Aponte et al., 2009). Widely used in pregnant women in malaria endemic zones (Briand et al., 2007; ter Kuile et al., 2007), IPT is now being contemplated for deployment in infants (IPTi) (Greenwood, 2007), in children (IPTc) (Buffet et al., 2008) and school children (IPTsc) (Clarke et al., 2008; Temperley et al., 2008; Barger et al., 2009). However, the possibility that this strategy may cause an increase of *P. falciparum* resistance to SP is a subject of debate (Sokhna et al., 2008; Dicko et al., 2010).

Point mutations in the genes encoding dihydrofolate reductase (*DHFR*) and dihydropteroate synthetase (*DHPS*), the respective targets of pyrimethamine (P) and sulfadoxine (S), have been associated with in vitro resistance to these drugs (Peterson et al., 1990; Wang et al., 1997). Increasing resistance to P is seen with the *DHFR* mutations S108N, N51 I and/or C59R, and I164L. A quintuple mutation, *DHFR* 108, 51, 59, and *DHPS* 437 and 540, was found to be predictive of clinical failure in Malawi (Kublin et al., 2002).

During the life cycle of Plasmodia a small percentage of the merozoites differentiate into sexual forms (gametocytes). Early stage I and II gametocytes appear after a few cycles of asexual multiplication, by the eighth day in in vitro cultures. Their maturation process, which includes stages III – VI, follows and peaks at around day 12 of culture. Stage V mature gametocytes are reached by day 14 of culture (Ponnudurai et al., 1982). When ingested by a mosquito, male and female gametocytes can unite, forming a zygote, which matures into the ookinete. The ookinete further differentiates into an oocyst, which will eventually release sporozoites. These sporozoites migrate in the mosquito's salivary glands, thus completing the lifecycle. The ability of the host to infect the mosquito vector is determined by the presence of infectious gametocytes and the gametocytes' sporogonic capacity.

Several anti-malarial drugs are known to induce *P. falciparum* gametocytes both in vivo and in vitro, (Buckling et al., 1999) which are suggestive of a response to drug-related stress. SP treatments increase the rate of gametocyte carriage in treated patients compared with untreated controls (Robert et al., 2000a,b; von Seidlein et al., 2001a).

Drug-resistant parasites are believed to be selected by drug pressure. Studies in South America found that in an area with rare SP failure, the presence of resistance-conferring mutations at the time of treatment was associated with prolonged parasite clearance times and with higher rates, levels and duration of gametocytemia (Mendez et al., 2002). Although some studies found a high infectivity of post-SP gametocytes (Targett et al., 2001), most published data and our own preliminary findings suggest that post-SP gametocytes present a

decreased infectivity (Robert et al., 2000a). The mechanisms involved in the low infectiousness of post-SP gametocyte are poorly understood.

Deterring the spread of anti-malarial drug resistance will require an understanding of the specific mechanisms involved. The objective of this study was to investigate the impact of SP on the biology of *P. falciparum* gametocytogenesis, gametocyte maturation, oocyst formation in *Anopheles* mosquitoes, and on mosquito viability. We confirm that S, P and SP induced gametocytogenesis, notably in mutant parasites. However, both drugs inhibit gametocyte maturation in both mutant and wild-type parasites. Furthermore, a high dose of S is toxic for *Anopheles stephensi*.

2. Materials and methods

2.1. Standard membrane feeding assay

Gametocyte culture (7.5 ml) was harvested and added to 10 Eppendorf centrifuge tubes, each with 380 μl of packed cells, and centrifuged (20 s, 20,000 *g*). The supernatant was removed, pellets were resuspended with 75 μl non-immune serum and collected in a 15 ml tube. The compounds to be tested were diluted in 90 μl non-immune human serum which were mixed with 180 μl of gametocyte suspension.

The suspension was fed to 3–5 days old mosquitoes. Six days after the feed, 10–20 mosquitoes per feeder were dissected and checked for oocysts after staining the stomach with 2% Mercurochrome (Ponnudurai et al., 1989a).

2.2. Determination of the dhfr and dhps genotypes

Laboratory adapted *P. falciparum* strains (NF-54 (Ponnudurai et al., 1981), NF-114, NF-135, NF-151 , 3D7 (Rosario, 1981), 7G8 (Burkot et al., 1984) and Dd2 (Wellems et al. ,1990)) were screened for inclusion in the study. These strains were selected because they were routinely grown and used for gametocyte production in the laboratory at Nijmegen, The Netherlands.

Parasitemic blood from cultures of each strain was spotted onto 3MM Whatman filter papers in the Nijmegen laboratory and sent to the Malaria Research and Training Center (MRTC) in Bamako, Mali for genotyping. DNA was extracted from these filter paper blots as previously described (Djimde et al., 2001). Nested mutation-specific PCR and nested PCR followed with restriction endonuclease digestion were used to determine the alleles of *dhfr* at codons 51, 59 and 108 and *dhps* at 437 and 540 as described (Djimde et al., 2008).

2.3. Gametocyte production

Frozen *P. falciparum* samples were thawed and grown in vitro to steady state in an automated tipper system in red blood cells (RBCs) of blood group O and non-immune A serum, and harvested after 13 or 14 days (Ponnudurai et al., 1982a). Subcultures at 0.5%, 1% or 3% parasitemia were grown in a shaker (Ponnudurai et al., 1983), a tipper (Ponnudurai et al., 1982a) or a candle jar (Ifediba and Vanderberg, 1981) at 5% hematocrit, respectively. The culture medium (RPMI, HEPES, hypoxanthine, human serum and bicarbonate) was changed twice each day without adding fresh RBCs. The cultures were maintained at 37° C to avoid any exflagellation. Thin smears were made regularly, Giemsa-stained and examined to monitor gametocyte presence and age. The infectivity of gametocytes was determined by a pre-feed experiment (van der Kolk et al., 2005).

2.4. Capacity to exflagellate

The capacity to exflagellate was tested for each culture containing mature stage V gametocytes prior to mosquitoes feeding (Ponnudurai et al., 1982b). The quality of mature gametocytes was assessed by testing the ability of the males to exflagellate. All materials and reagents were at 37 ° C before starting the experiment. Approximately 20 ul of culture were centrifuged and 2 ul of pellet were mixed with a drop of FCS on a slide. The slide was then put in a humidified box at room temperature and covered for 10 min. The preparation was covered with a cover-slide, sealed with Vaseline and observed under a light microscope. The exflagellated male gametocytes app eared as small moving RBCs but at higher resolution flagella could be observed.

2.5. Drug treatment of parasite culture

The parasite cultures were treated with different concentrations of S and P at different stages of gametocyte development. These concentrations were chosen to correspond with physiological serum levels of S or P on days 3, 7, 14, 21 and 28 following a single oral dose of SP. We hereafter refer to these concentrations as SD3 (sulfadoxine day 3 = 61 ug/ml), SD7 (sulfadoxine day 7 = 33.8 ug/ml), SD14 (sulfadoxine day 14 = 14.2 ug/ml), SD21 (sulfadoxine day 21 = 7.6 ug/ml) and SD28 (sulfadoxine day 28 = 3.8 ug/ml); PD3 (pyrimethamine day 3 = 154.7 ng/ml), PD7 (pyrimethamine day 7 = 66.6 ng/ml), PD14 (pyrimethamine day 14 = 15.7 ng/ml), PD21 (pyrimethamine day 21 = 19.7 ng/ml) and PD28 (pyrimethamine day 28 = 9.9 ng/ml); and SPD3 (sulfadoxine-pyrimethamine day 3), SPD7, SPD14, SPD21 and SPD28 (our unpublished data).

The following experimental design was based on the biology and timing of the different steps in gametocytogenesis.

2.5.1. Effect of SP on the induction of gametocytes—Four days old parasite cultures of NF135 and NF54 were transferred on 24-well plates and submitted to SD3, SD7, SD14, SD21, SD28 or PD3, PD7, PD14, PD21, PD28 for 5 days (Fig. 1A). No drug was added on subsequent days of culture. Gametocytogenesis and gametocytemia were assessed by light microscopy from day 8 onwards. The quality (morphology, pigmentation, overall appearance) and infectivity of the resulting gametocytes were assessed when they reached 14 days of age.

2.5.2. Effect of SP on gametocyte maturation—Eight days old cultures containing early stage II gametocytes of NF135 and NF54 were transferred on 24-well plates and submitted to SD3, SD7, SD14, PD3, PD7 and PD14 (Fig. 1B). Drug pressure was maintained for 5 days after which time normal culture medium was continued. The quality (morphology, pigmentation, overall appearance) and infectivity of the resulting gametocytes were assessed at day 14.

2.5.3. Effect of SP on gametocyte infectivity—Gametocyte production cultures started with the two strains in human RBCs and were maintained for 14 days in a shaking culture system for NF135 and the static culture system for NF54. Standard membrane feeding assays (SMFAs) with laboratory reared *A. stephensi* were performed as previously described (Ponnudurai et al., 1989a). Briefly, a pre-feed was first performed on a small scale as previously described (Ponnudurai et al., 1989a); 300 ul of culture containing 14–16 days old gametocytes were mixed with 180 ul of RBCs and centrifuged. The supernatant was carefully discarded and the pellet mixed with 150 ul of human serum or FCS. This gametocyte preparation was transferred to a small feeder and fed to 20 *A. stephensi* mosquitoes which had been starved for 12 h. All operations were performed at 37° C.

Twenty-four hours after the feeding, 10 mosquitoes were checked for the presence of round forms using FITC-conjugated anti-Pfs25 monoclonal antibodies.

The SMFA was conducted the next day only f round forms were found in the midgut of 20 dissected mosquitoes. Furthermore, 30 mosquitoes were allowed to feed on each sample but 20 surviving mosquitoes were dissected (Ponnudurai et al., 1989b). Reduction of infectivity is defined as a significant decrease in the prevalence of oocyst positivity among the dissected mosquitoes. Between days 14 and 16, stage V mature gametocytes were tested for exflagellation before proceeding with a SMFA.

S, P and SP were mixed with the gametocyte preparation to yield final concentrations equivalent to SD3, SD7, SD14, PD3, PD7, PD14, SPD3, SPD7 or SPD14 (Fig. 1C). The resulting preparations (mature gametocytes + drug) were used to feed mosquitoes (*A. stephensi*) through a membrane feeder. For each of three consecutive experiments 20 mosquitoes per strain were dissected on day 8 post-feeding and the presence and the density of oocysts were assessed by light microscopy.

2.6. Mosquito survival

As described in Section 2.1, during the infectivity SMFAs 30 mosquitoes in a cup were fed on gametocyte preparations containing no drug or various concentrations of drugs. Twenty-four hours after the feeding experiments, the number of surviving mosquitoes were counted and recorded for each experiment and each drug concentration.

2.7. Data handling and analysis

The results were recorded on data sheets and entered in Microsoft Access and further analyzed in SPSS vs.12.0. Descriptive statistics provided gametocyte prevalence, gametocyte loads, mosquito oocyst infection rates and loads. A Chi-square test was used to compare prevalence of gametocytes and oocyst infection rates, between comparison groups. Two-way ANOVA or Kruskal-Wallis H tests were used to compare the spread of the oocyst loads between the comparison groups. Experiments were repeated at least once in the same facility, by the same investigators.

3. Results

3.1. Genotyping results

The *dhfr* and *dhps* genotypes of the full set of strains analyzed are presented in Table 1. Two strains with the most contrasting genotypes, NF135, (quintuple mutant for *dhfr* N51I+C59R +S108N and *dhps* A437G+K540E) and NF54 (wild-type for all but mutant for *dhps* A437G) were selected for all subsequent gametocyte production and feeding experiments.

3.2. Induction of gametocytes with different concentrations of S and P

With NF54 and both S and P, while the controls progressed to mature gametocytes (Fig. 2A and D), all drug concentrations lead to parasite degeneration. (Fig. 2B and C).

In contrast, incubation of asexual stages of NF135 with different concentrations of S and P between days 4 and 8 induced differentiation into gametocyte stage II. These young gametocytes were observed within 24 h of drug treatment for the highest SD3 and PD3 drug concentrations and after 48 h for the lowest SD28 and PD28 drug concentrations. In the control untreated cultures, the first stage II gametocytes appeared on day 8 of culture. The resulting stages IV and V of NF-135 gametocytes showed no differences compared with control cultures. Exflagellation tests were positive for all drug concentrations and the controls. No SMFAs were done with these cultures.

3.3. Gametocyte maturation under S and P pressure

Day 8 early gametocytes of both NF 54 and NF-135 were exposed to the different concentrations of S and P. After 5 days of drug treatment, the resulting mature gametocytes of both strains were atypical. They were smaller, the cytoplasm showed darker and fatter pigments and the extremities were sharper (Fig. 2E and F) compared with control gametocytes (Fig. 2D). These gametocytes were neither able to exflagellate nor to infect mosquitoes.

3.4. Effect of S, P and SP on gametocyte infectivity

Since control NF135 cultures did not produce sufficient numbers of gametocytes, data are shown for NF54.

NF54 produced mature, healthy gametocytes by day 14 in culture (Fig. 2D). All exflagellation tests were positive and control SMFA experiments were successful (Table 2). In a typical experiment (Table 2) physiological Day 3 concentrations of S, P and SP significantly reduced the prevalence of oocysts in *A. stephensi*. SD7 and SPD7 equally significantly decreased oocyst positivity in the fed mosquitoes while P alone had no effect. Day 14 and lower concentrations had no effect on oocyst numbers. As shown in Table 2, most experiments were repeated once or twice on different days but results remained consistent.

Similarly, SD3, SD7, SD14, SPD3, SPD7 and SPD14 concentrations significantly decreased the density of oocysts (Fig. 3A and C). With P alone, the highest PD3 concentration significantly decreased oocyst density compared with the controls; PD7 concentration showed a trend towards decreased oocyst density but it did not reach statistical significance ($P = 0.09$). The densities of oocysts with PD14 were similar to controls (Fig. 3B)

3.5. Effect of S, P and SP on mosquito viability

The number of surviving mosquitoes was counted 24 h after membrane feeds containing gametocytes and the different concentrations of S and P. In three separate experiments, SD3 concentrations killed an average of 43.3% (95% Confidence Interval (95% CI): 33.3 – 53.3; $n = 90$) of the fed mosquitoes (Fig. 4). The addition of P further increased mosquito death rate to an average of 51.7% (95% CI: 39 – 65; $n = 60$) (Fig. 4). There were no mosquito deaths with P alone nor with the any other drug concentrations (data not shown).

4. Discussion

We showed that S and P have complex effects on the biology of gametocytogenesis. Both drugs induce differentiation of asexual forms into sexual forms, initiating gametocytogenesis. However, treatment of young gametocytes with both drugs impaired their further development into mature stage V gametocytes. The data is consistent with a scenario where the parasites react to the stress imposed by the drugs by committing to their sexual stages. Yet, the drug maintained some of its toxic effect on the newly formed sexual stages leading to odd mature gametocytes. These effects were dose-dependent.

When added to a blood meal together with otherwise healthy and mature gametocytes, S and P significantly reduced the infectivity of the gametocytes to *Anopheles* mosquitoes. This effect was also dose-dependent since the rate of decrease was higher for the highest dose tested (day 3 drug levels) than for the lower day 7 drug level concentrations. When day 14 drug level concentrations were tested, there were no more differences from controls. Taken together, the above results support previous studies including our own recent report where a single oral dose of SP yielded a sharp increase in gametocyte carriage in the treated patients,

but the resulting gametocytes had very low infectivity for *Anopheles gambiae* by direct feeding (Beavogui et al., 2010). Our data are also consistent with analogous experiments performed in the 1950s with P, suggesting that the low rate of infectivity of post-treatment gametocytes may be due to a sporontocidal effect of the drug that is ingested together with gametocytes during the mosquitoes' blood meals (Jeffery, 1958a). The effect of drugs on infectivity appeared to be dose-related (Shute and Maryon, 1954; Young and Burgess, 1957a). Furthermore, the sporontocidal effect of P appears to be dependent on the rate of efficacy of the drug (Jeffery, 1958b; Young and Burgess, 1957b). Hogh et al. (1998) found that SP decreased infectivity .

By contrast the effect of S on gametocyte infectivity has been much less studied (Laing, 1965). This may be due to the fact that S and sulfa-drugs were almost never widely used alone for the treatment of malaria in the field. Therefore, our study provides one of the first indications that S could also have a sporontocidal effect. Gametocyte sex ratio is important for effective transmission (Sowunmi and Fateye, 2003a,b) but the nature of the drug does not appear to influence the sex ratio of post treatment gametocytes (Robert et al., 2003; Talman et al., 2004).

The highest day 3 S concentration was lethal to the fed mosquitoes as survival rates were significantly reduced compared with the no drug controls. The same level of lethality was observed when S and P were fed to mosquitoes together with the gametocyte preparation. This shows that, although P alone has no effect on mosquito survival, the addition of P to S tended to increase the lethal effect of S. The mosquitoes were fed with drug and gametocytes and the observed lethal effect could be due to the concomitant presence of both the parasites and the drug. However, it is known that the presence of the gametocytes alone does not kill the mosquitoes and the lethality was only measured 24 h after feeding. Taken together, the data suggest that a significant number of mosquitoes that feed on patients between days 1–3 post-SP treatment would not survive.

When treated with SP, the sensitive NF54 parasites were killed as expected, while the NF-135- resistant parasites were stimulated into gametocytogenesis. Although some parasites died, there were enough asexual forms that could survive to differentiate into gametocytes. In the absence of continuous drug pressure, the resulting gametocytes matured into normal mature gametocytes capable of exflagellation. This is consistent with previous reports showing that mutant/resistant parasites have a longer parasite clearance time which may favor differentiation into gametocytes (Mendez et al., 2002; Tjitra et al., 2002; von Seidlein L et al., 2001b; Ali et al., 2006; Barnes et al., 2008). SP treatment is known to select *dhfr* and *dhps* mutant asexual parasites (Tekete et al., 2009) and gametocytes (Beavogui et al., 2010).

This study demonstrates that although SP induces gametocytogenesis, it also kills gametocytes and the malaria vector *A. stephensi*.

Acknowledgments

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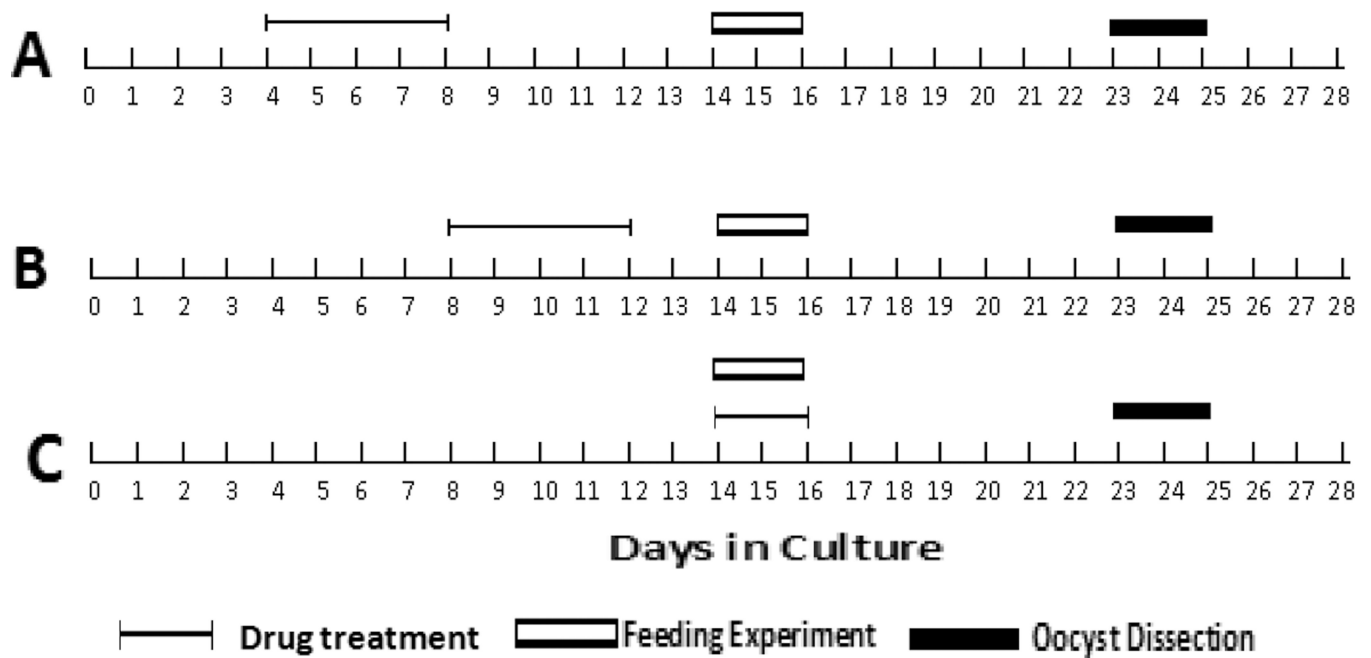
Fig. 1.
Sulfadoxine, pyrimethamine or sulfadoxine-pyrimethamine pressure, mosquito feeding and oocyst measurement days are shown for each experimental design. A) Impact of Sulfadoxine, pyrimethamine or sulfadoxine-pyrimethamine on gametocytogenesis induction; B) impact of Sulfadoxine, pyrimethamine or sulfadoxine-pyrimethamine on gametocyte maturation; C) impact of Sulfadoxine, pyrimethamine or sulfadoxine-pyrimethamine on gametocyte infectivity.
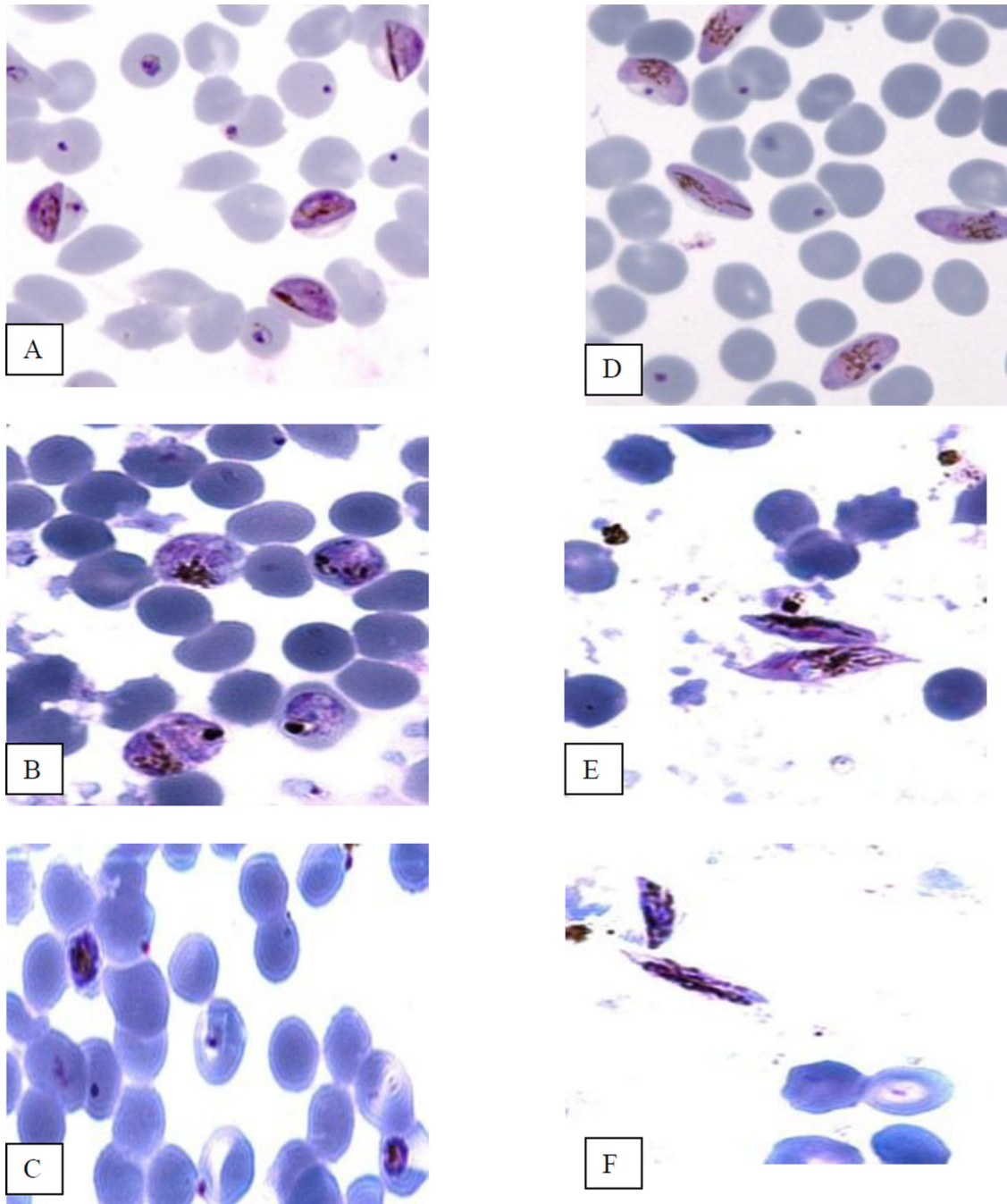
Fig. 2.
Effect of sulfadoxine and pyrimethamine on *Plasmodium falciparum* NF 54 strain. A–C) Gametocyte induction experiment. A) Stage II normal gametocytes at day 8; B) cultures treated with pyrimethamine; C: cultures treated with sulfadoxine. D–F) Gametocyte maturation experiment. D) Stage V normal gametocyte at day 14; E) Stage V gametocytes obtained after treatment with pyrimethamine; F) Stage V gametocytes obtained after treatment with sulfadoxine.
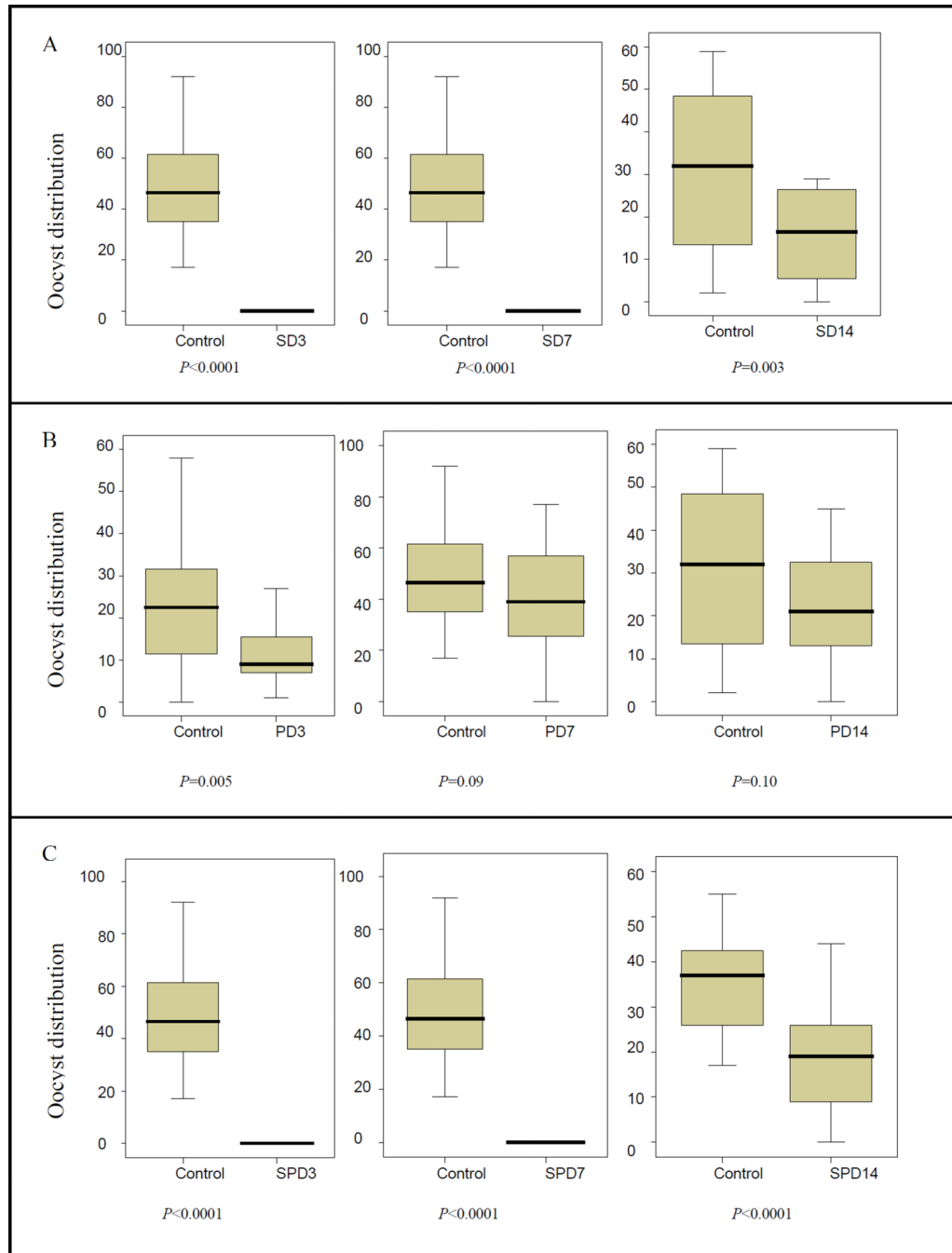
Fig. 3.
Effects of various concentrations of drugs on *Plasmodium falciparum* oocyst density. A)
Effects of different Sulfadoxine concentrations on oocyst distributions in mosquitoes. B)
Effects of different Pyrimethamine concentrations on oocyst distributions in mosquitoes. C)
Effects of different Sulfadoxine462 pyrimethamine concentrations on oocyst distributions in
mosquitoes. S, sulfadoxine; P, pyrimethamine; SP, sulfadoxine-pyrimethamine; SD3 =
sulfadoxine concentration equivalent to serum levels of sulfadoxine at day 3 following an
oral dose of sulfadoxine-pyrimethamine; SD7, sulfadoxine concentration equivalent to
serum levels of sulfadoxine at day 7 following an oral dose of sulfadoxine466
pyrimethamine; SD14, sulfadoxine concentration equivalent to serum levels of sulfadoxine

at day 14 following an oral dose of sulfadoxine-pyrimethamine; idem for PD3, PD7, PD14, SPD3, SPD7 and SPD14.
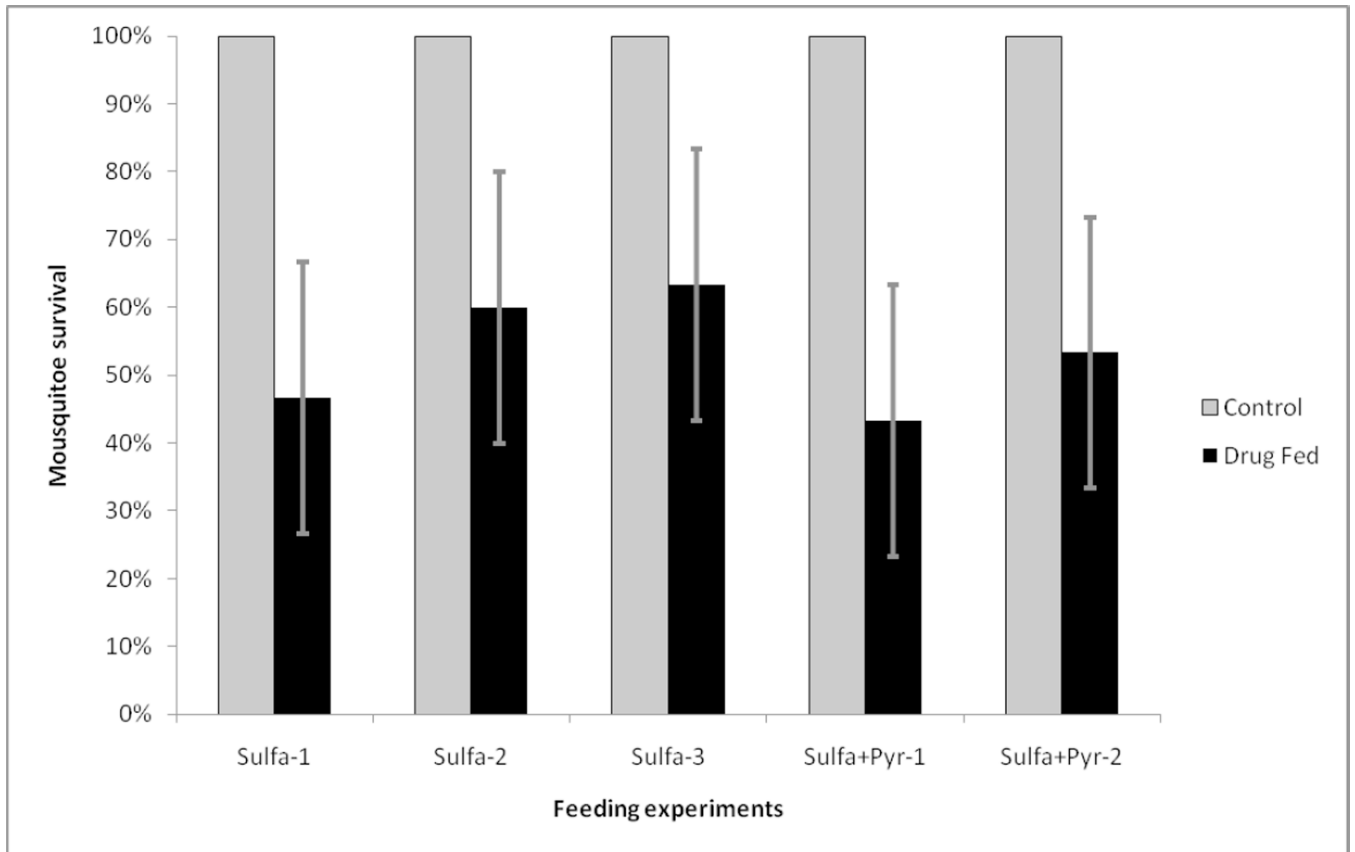
Fig. 4.
Survival of mosquitoes fed with the highest day 3 concentrations of sulfadoxine (three experiments are shown) or sulfadoxine-pyrimethamine (two experiments are presented).

Table 1

Dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genotypes of *Plasmodium falciparum* laboratory strains screened during this study.

Codons/*P. falciparum* strains	*dhfr*			*dhps*		
	51	59	108	437	540	
NF-135-W45	M	M	M	M	M
3D7	W	W	W	M	W
7G8	M	W	M	M	W
NF-135-T8	M	M	M	M	M
NF-151	M	W	M	W	W
NF-135-T7	M	M	M	M	M
NF-114	W	M	M	W	W
NF-54	W	W	W	M	W
Dd2	M	M	M	M	W

M, mutant allele

W, wild-type allele.

Table 2

Proportion of oocyst positivity in *Anopheles stephensi* mosquitoes fed on mature gametocyte preparations in the presence of physiological concentrations of sulfadoxine, pyrimethamine or sulfadoxine-pyrimethamine

Experiment Number	Control			Sulfadoxine			Pyrimethamine			Sulfadoxine-Pyrimethamine		
	1	2	3	1	2	3	1	2	3	1	2	3
Oocyst + mosquitoes,% (*n*)												
Day 3 concentrations [a]	55(20)	95(20)	100(20)	0(14) [b]	11(18) [b]	0(19) [b]	5(20) [b]	100(20)	40(20) [b]	0(13) [b]	0(16) [b]	N
Day 7 concentrations	100(20)	100(20)	N	0(20) [b]	5(20) [b]	N	95(20)	85(20)	N	0(20) [b]	N	N
Day 14 concentrations	100(20)	100(20)	N	85(20)	N	N	90(20)	N	N	95(20)	N	N

[a] Each value is compared to the control value of the same feeding experiment.

[b] *P* < 0.001

N = experiment not done.