REDUCING MALARIA TRANSMISSION: THE EPIDEMIOLOGY AND TREATMENT OF *PLASMODIUM FALCIPARUM* GAMETOCYTEMIA

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Epidemiology.

Chapel Hill
2012

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ABSTRACT

NAMAN KIRIT SHAH: Reducing Malaria Transmission: The Epidemiology and Treatment
of *Plasmodium falciparum* Gametocytemia
(Under the direction of Steven R Meshnick, MD, PhD, and Neena Valecha, MD)

Gametocytes are the sexual stage of the *Plasmodia* life cycle which render malaria

cases infectious to mosquitoes. The proportion of *P. falciparum* malaria cases with

gametocytemia and the duration of gametocytemia are varied. Interventions for detecting and

treating gametocytemia also differ from those used against asexual parasitemia. In areas of

low transmission, such as most of India, the size of the infectious reservoir drives

transmission. The purpose of this dissertation was to 1) determine the epidemiology and risk

factors for gametocytemia in order to better target interventions, and 2) estimate the effect of

primaquine in addition to artesunate plus sulfadoxine-pyrimethamine (AS+SP) to guide

policy for reducing post-treatment malaria transmission. Using data from therapeutic efficacy

studies conducted through the National Antimalarial Drug Resistance Monitoring System

from 2009 to 2010, we measured the prevalence of gametocytemia in relation to various

clinical and demographic factors. We found that all age groups, including adults, contribute

substantially to the reservoir for potential transmission. We identified four risk factors –

younger age group, previous antimalarial drug intake, sex, and region – from which we

created a clinical algorithm for predicting gametocytemia. The predictive power of the model

was low, suggesting the need for a universal approach for anti-gametocyte interventions. We

compared trial sites which used primaquine to sites which did not to estimate the additional

effect of primaquine. AS+SP with primaquine increased the rate of gametocyte clearance, prevented the development of new gametocytemia, and reduced the area under the gametocyte density over time curve over the study follow-up compared to AS+SP alone. Primaquine was well tolerated and no serious adverse events were reported. Adding primaquine to AS+SP for the treatment of *P. falciparum* infection in India would decrease the potential for post-treatment malaria transmission.

"Now it is to these gametocytes that an extreme interest attaches, because it is to them, and to Manson's study of them, that we owe the solution of the malaria problem."

- Sir Ronald Ross (1)

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ACKNOWLEDGEMENTS

I cannot fully express the gratitude I feel for all the help and support I have received during my doctoral work. The debts I owe are many and I fear, can never be fully repaid - the best I can hope for is to start paying them forward.

First and foremost, I must thank my committee. Steve has guided my academic career since I started working with him in 2005 while in college. His support for students and their work internationally is inspiring – he sent me to both Phnom Penh (my first international malaria work) and to Jabalpur (my first India malaria work). Steve gave me the independence I needed to seek out my own projects, and my own growth, but I may have abused that freedom occasionally with my hard headed nature. Seven years of mentoring me can only be a labor of love! Charlie is the best teacher I have ever come across. If wisdom is the ability to balance then he is indeed wise always finding the appropriate mix of methods and public health, of science and ideology. He is a credit to, and I would say foundational for, our school. I could not imagine my training without him. In the oft, isolated world of academia, Pia was a respite with her rootedness in the practice of public health. She understood the PhD was just a means to larger ends and helped me navigate the gap I perceived between those two worlds. Every student needs such a champion. Jon, as the youngest member of the group, has in turn reminded me that serious work is compatible with serious humor. As importantly, his deep clinical knowledge and careful readings improved the work. Jon has been a sounding board for me long before the dissertation and he is a great example of what

physician-scientists can achieve. Allan is a rare breed, one of the handful of remaining malariologists in the world. To top it off he has intimate knowledge of India and all her complexities without which I could not have built my assessments of the policy and program operation implications that I strived for in my work. I am lucky to have had his active participation during his well-earned retirement which included calls at 2am from the Philippines for him. Finally, Neena warmly welcomed me into her institute and by extension the arena of national research and decision-making. The amazing part is she did so without any precedent for such action, deliberately taking a risk on me with a bigger vision in mind. She is a true leader and has taught me both science and administration well.

My work has been split between two continents and in fact, two homes. India has been the key to my professional and personal learning, mostly as a product of the people I've been fortunate to interact with. A previous remark bears repeating, "I would also like to acknowledge other colleagues in India, with whom I have learned, and continue to learn, the difficult task of improving malaria control". Neeru Singh invited and supported me, sight unseen, to a terrific conference in Jabalpur which opened subsequent doors. I am happy to have had the chance to return there and see her several times since. Krongthong Thimasarn allowed me to intern with her at WHO-SEARO, sharing the joys and pains of her office, as well as instructive stories and her considerable experience. This platform allowed me to learn about international cooperation and inserted me into the midst of the close knit malaria community in India. GPS Dhillon, GS Sonal, Usha Arora and AC Dhariwal have over the years hosted me in the NVBDCP directorate, given me meaningful work, and in doing so explained and demonstrated the hard work and tough decisions national programme managers face. It has been a privilege which I hope to continue. Neelima Mishra and Anup Anvikar have been blessings at NIMR – one could not ask for more supportive, hard-working

scientists, who while much senior to me were always kind enough to treat me as a colleague. Bina Srivastava and Jai Prakash Singh provided invaluable and kind technical support. Jagannath Sarkar, SK Sharma, PK Tyagi, and HC Srivastava not only received me in their offices, joined me in field work, and developed my abilities but took care of me also - we broke bread together.

The doctoral process is much more than the dissertation. Yvan Hutin is my role model for how to conduct oneself as a public health worker. He is responsible for my trajectory and my broad exposure to different institutions. He stepped out of the way to bring me to learn from his work in Beijing and Stockholm. Since our chance meeting many years ago, Yvan has been an incredible mentor and friend. Kevin Baird has generously corresponded with me about malaria for many years even though we had never met until last fall. His replies were always long, funny, instructive, and always encouraging. I took Steve Wing's seminar and which pushed my understanding of our field farther than any other experience. He has since continued to help me think about ethics and justice in public health through my writings and personal experiences. Joseph Cook took a great interest in my progress and always lent a kind ear. He introduced me to T Jacob John, an inspiring pioneer of public health in India who critically, has helped me crystallize many nebulous sentiments into a concrete goal for the future. Science should be marked by collaboration and sharing and Brooke Hoots reinforced this culture by providing me with an invaluable copy of her work prior to its publication.

I could not have completed my work without the support of Nancy Colvin, Carmen Woody, Alison Regan, and Carol Herion. They are all-stars and in fairness, more than support it was their patience which was essential. Same goes for all the members of the Meshnick lab with special mention to Jessica Lin for many interesting discussions. The MD/PhD program at

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LIST OF ABBREVIATIONS

ACT	Artemisinin Combination Therapy
AS	Artesunate
AUC	Area Under the Curve
BQ	Bulaquine
CI	Confidence Interval
CQ	Chloroquine
DDT	Dichloro-Diphenyl-Trichloroethane
EMM	Effect Measure Modification
FN	False Negative
FP	False Positive
GEE	Generalized Estimating Equations
GoI	Government of India
HR	Hazard Ratio
IQR	Interquartile Range
IRR	Incidence Rate Ratio
MoHFW	Ministry of Health and Family Welfare
MPO	Modified Plan of Operations
NAMP	National Anti-Malaria Programme
NIMR	National Institute of Malaria Research
NMCP	National Malaria Control Programme
NMEP	National Malaria Eradication Programme
NVBDCP	National Vector Borne Disease Control Programme

POR	Prevalence Odds Ratio
PQ	Primaquine
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic
RS	Risk Score
SD	Standard Deviation
SE	Standard Error
SEARO	Southeast Asia Regional Office
SP	Sulfadoxine-pyrimethamine
WBC	White blood cell
WHO	World Health Organization

CHAPTER ONE: SPECIFIC AIMS

Gametocytes are the sexual stage of *Plasmodia* life cycle which render malaria cases infectious to mosquitoes and propagate transmission. The proportion of *P. falciparum* malaria cases with gametocytemia and the duration and density of that gametocytemia are varied. In areas of low transmission, such as most of India, the number of infective hosts, as opposed to vectorial capacity, determines transmission. Thus, studies of the epidemiology of gametocytemia are needed to better define this key reservoir. Interventions for detecting and treating gametocytemia also differ from those used against asexual parasitemia. So an improved understanding of gametocytes opens the possibility of distinct transmission-blocking control strategies for the nation.

First, which case-patients carry gametocytes? Universal application of gametocytocidal interventions adds cost and may expose some individuals to potentially severe side effects. Targeting interventions by direct examination of gametocytes during routine microscopy can be difficult due to low density, poor training, and the heavy workload of the microscopist. Easily discerned risk factors associated with gametocytemia could help but these have not been assessed in Indian transmission settings where, in comparison to sub-Saharan Africa, natural immunity is low, many patients are adults, and the presence of competing malaria species may be an important determinant. Risk factors used in a clinical risk score could enable field-level screening.

Second, how should we treat gametocytemia? Artemisinin combination therapies (ACT), the first-line treatment in most countries including India, eliminate immature gametocytes but not mature gametocytes which may persist for up to one month post-treatment. The key operational question is whether a single dose of primaquine, which is inexpensive and effective against mature gametocytes, should be added to artemisinin combination therapies to reduce the potential post-treatment transmission of the infection. Currently, we have little data regarding the safety or effectiveness of doing so.

This dissertation aims to describe the epidemiology of gametocytemia in India, including its prevalence, age-structure, and risk factors, as well as estimate the effect of primaquine in addition to an ACT for improving the control of malaria in the country by reducing potential transmission. We used data from the National Antimalarial Drug Resistance Monitoring System, run by the National Institute of Malaria Research (NIMR) and the National Vector Borne Disease Control Programme (NVBDCP), which conducted therapeutic efficacy trials at 21 sentinel sites during 2009 and 2010, where 9 sites used primaquine in addition to artesunate plus sulfadoxine-pyrimethamine (AS+SP) while 12 used AS+SP alone.

Specific Aim 1

Determine which *P. falciparum* patients carry gametocytes. We will compare the prevalence of gametocytemia among the clinical and demographic factors available in routine settings (i.e. age, gender, asexual parasite density, current fever, previous antimalarial drug intake, region, etc.) and develop a risk score for detecting gametocytemia

Rationale:

Identifying the type of person likely to carry gametocytes may enable us to target individuals for screening and/or treatment without the need for diagnostic equipment. Conversely, an inability to target accurately would suggest the need for universal application of interventions.

Specific Aim 2

Estimate the effect of adding primaquine to artemisinin combination therapy on post-treatment clearance (gametocyte survival) and post-treatment carriage (gametocyte incidence) during 28 days of follow-up

Rationale:

Measuring the effectiveness of primaquine will provide the first quantitative data on the transmission blocking role of primaquine in combination with an ACT in India and guide the national drug policy.

CHAPTER TWO: BACKGROUND AND SIGNIFICANCE

MALARIA AS A PUBLIC HEALTH PROBLEM

Malaria is the resultant febrile illness from an infection by a parasite of the *Plasmodium* genus. *Plasmodium ovale*, *P. malariae*, *P. vivax*, and *P. falciparum* represent the four main species which infect humans (2); the latter two are responsible for the vast majority of disease burden and most fatal cases are attributed to *P. falciparum* which can cause severe malaria. Health effects of malaria infection include debilitating illness such as severe anemia, jaundice, kidney failure, coma, and death (3). According to the World Health Organization, approximately 40 percent of the world is still at risk for malaria (4). An estimated 225 million clinical episodes and 0.8 million deaths due to malaria occurred in 2008 (4). However considerable uncertainty exists around the estimates, particularly fatal cases, both globally and applied to individual country levels (5). As with many tropical diseases, malaria risk is inequitable with approximately 58% of cases distributed among the poorest quintile of the world's population (6). Historical declines in malaria have in general been maintained only where development was associated with a reduction of the social and economic risk factors for the disease (7,8). Despite the existence of successful control interventions for over 100 years and the elimination of the disease from large parts of the world, malaria remains a grave public health concern.

STAGES IN THE MALARIA LIFE CYCLE

The malaria parasite has a complex life cycle traversing through both mosquito and human hosts (Figure 2.1). Within persons there is the liver stage of infection (exo-erythrocytic) where the infection begins, and in the case of *P. vivax* and *P. ovale* dormancy may persist, and a blood stage (erythrocytic cycle) which produces clinical symptoms (2). Gametocytes are the sexual stage of *Plasmodia* life cycle which render malaria cases infectious to mosquitoes and propagate transmission (9).

In *P. vivax* gametocytes appear in most cases and shortly after the emergence of asexual parasites from the exo-erythrocytic cycle (10). In *P. falciparum* however, the proportion of malaria cases with gametocytemia and the duration and density of that gametocytemia are varied (11). Furthermore, infective gametocytes[1] do not appear until later in the blood-stage infection nearly several days after patency (the first appearance of parasites in the blood stream) and multiple cycles of asexual parasitemia (12). The mean duration of survival for a mature gametocyte is between 4-6 days (13,14) (Figure 2.2 A). Coupled with the maturation and progressive release of sequestered gametocytes, gametocytemia may persist for three to six weeks after the elimination of its precursor asexual stages (by successful treatment for example) however the viability of gametocytes towards the end of this period, that is their ability to infect mosquitoes, is less certain (13,15,16) (Figure 2.2 B).

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[1] From here on gametocyte or gametocytemia refers to those of *P. falciparum only*

EPIDEMIOLOGY OF GAMETOCYTEMIA

Although gametocytes are central to understanding transmission, few studies in the past 50 years have tried to demarcate the infectious reservoir (15). This may be because in high transmission areas of interest, namely sub-Saharan Africa which has long dominated the donor perspective given its high burden and generally poor health systems, the infectious reservoir is not an important determinant of the intensity of transmission relative to high vector competence (17). However, in low transmission areas, where the vector is not as efficient, the proportion of infectious hosts is more critical to the maintenance of endemicity (17). With recent reductions in malaria transmission due to intervention scale-up, and increased focus on malaria elimination worldwide, interest in the epidemiology of gametocytes is gaining traction with the hopes that a better understand will help reduce the remaining infectious reservoir (18–20).

Microscopically detected gametocytemia is typically present in less than 50% of cases (21) though the proportion rises greatly using molecular techniques, albeit at lower densities (13). The overall density of gametocytes among the total parasite population tends to be low, typically less than 5% of parasites (22,23). A key aspect of gametocyte epidemiology is age related trends due to immunity (24). In high transmission settings, the proportion of infections with gametocytemia is higher in children (25,26). It is unclear whether age directly affects gametocytemia or whether this is just a reflection of the higher overall parasite density, which leads to more gametocytemia, which is typically found in children (27). However, among individuals with gametocytemia, the density of gametocytes relative to total parasite density is higher in adults (28,29). In low transmission settings gametocytemia is thought to be more evenly distributed (26,30). There is limited evidence for seasonal differences (31–34) in gametocytemia though other factors such as longer

duration of clinical symptoms (30,35,36), being afebrile at the time of presentation (37), and previous antimalarial drug intake (38), and epidemic situations are associated with a higher prevalence of gametocytemia (39,40).

CONTROL OF GAMETOCYTES

The control of gametocytes is largely achieved through general malaria control. Treatment of parasitemia prevents the generation of new gametocytes and limits infectivity, while vector control measures, in addition to protecting individuals from new infections, prevents mosquito uptake of existing infections. Gametocyte-specific interventions are, at present, limited to its diagnosis and treatment which we discuss in more detail. In the future, preventative interventions such as transmission-blocking vaccines, which induce immunity against gametocytes or the mosquito stage gamete, may be available (41,42).

DIAGNOSIS OF GAMETOCYTEMIA

Typically gametocytes are diagnosed through light microscopy using the same methods as those for screening parasitemia. *P. falciparum* gametocytes, with their large crescents shape, are distinctive and easy to recognize. The detection limit for thick film microscopy of approximately 8-40 parasites per microliter (depending on whether 200-1000 WBC counted) poses a challenge though as gametocyte density is substantially lower than that of asexual parasites (typically <5% of the total population). Thus, microscopy is a specific tool but its sensitivity may vary. Coupled with poor training and heavy workloads, the diagnosis of gametocytemia in routine settings is difficult.

Recently, molecular methods that detect gametocyte-stage specific RNA transcripts have been employed (43–45). The measured prevalence with molecular tools ranged between

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2-4 times that detected by microscopy (46–51). However, not all gametocytemia is the same. While sub-microscopic density infections can infect mosquitoes, the probability of infection, the proportion of mosquitoes infected, and the density of infection in mosquitoes increases with increased gametocyte density (48,52). Comparisons of host infectivity must account for both the frequency and magnitude of gametocytemia.

TREATMENT OF GAMETOCYTEMIA

Most antimalarial drugs target blood-stage schizonts, responsible for clinical symptoms, and have varying activity against gametocytes. Chloroquine, quinine, and sulfadoxine-pyrimethamine are active against newly differentiated gametocytes but lose potency as gametocyte development progresses (53–57). In fact, with some of these drugs post-treatment increases in gametocytemia are often observed, attributed to increased "stress" acting through some unknown mechanism, inducing gametocytogenesis among remaining parasites to ensure survival. Artemisinin is a potent gametocytocidal drug against all stages except for those already fully mature (i.e. in peripheral circulation) and this characteristic contributed to the impressive declines in transmission following the widespread implementation of combination therapy in several countries, notably Thailand (58). However, remaining mature gametocytes (present in 10-30% of patients), remain infectious and persist for up to a month post-treatment and can therefore contribute to transmission (51,59–62).

Primaquine, which has no activity against asexual *P. falciparum* parasites, is effective against mature gametocytes but its activity against immature gametocytes is less certain (63–65). A single-dose of primaquine has been used as an as an adjunct treatment alongside first-line therapies, for its gametocytocidal effect, in many countries since the 1960s (66–70). One

challenge with primaquine is the concern over its safety in G6PD deficient patients and others where the drug can precipitate mild to severe hemolysis (71–73). However, the safety of a single dose, rapidly eliminated, is thought to be greater than the 14 day anti-relapse treatment provided in *P. vivax* treatment which was the principal cause of safety concerns (74). The ethics for single-dose primaquine have been questioned in light of these risks and the community rather than individual level benefit conferred (74).Studies of artemisinin combination therapies (ACT) with primaquine indicate reductions in gametocyte carriage, area under the curve, clearance time, and infectivity, whether assessed microscopically or by molecular techniques, relative to ACT alone (66–70). The magnitude of the effect appears to vary though with reduction in post-treatment gametocyte carriage of 25-75% among African children, 60% in Colombian adults, and 92% in Burmese patients of all ages (47,75,76).

MALARIA IN INDIA

The Republic of India represents the largest national population at risk for malaria in the world with 85% of the populace residing in malarious zone (77,78) (Figure 2.3). Both major *Plasmodium* species, six primary malaria vectors, several ecotypes including urban malaria, and transmission intensities ranging from unstable to hyper endemic highlight the challenging malaria epidemiology in the nation (79). At the time of independence in 1947, the Indian people were suffering from an estimated 75 million cases and 800,000 deaths per year (80). After independence health care was prioritized and the control of malaria was one of the key aims of the Government of India (GoI). In 1953, the National Malaria Control Programme (NMCP) was launched under the Ministry of Health and Family Welfare (MoHFW) and protected a population of approximately 165 million with DDT spraying (81).

The control programme evolved into the National Malaria Eradication Programme (NMEP) in 1958 (82). Reliable surveillance gradually developed during the eradication period (83) and the programme seemed to be highly effective with only 99,667 malaria cases and zero deaths reported in 1965 (84).

However, the long-term success of malaria control could not be sustained (85). Increasing insecticide resistance in mosquitoes, urbanization, population migration, integration with the general health services, financial difficulties and other operational challenges laid the foundation for a resurgence of malaria (84,86). In 1976, malaria cases reached a post-eradication peak of 6.47 million cases (87). A new strategy, the Modified Plan of Operation (MPO), was introduced in 1977 (88) after which there has been a steady decline in malaria cases in the country (Figure 2.4) (89). In 1998 the NMEP was re-christened the National Anti-Malaria Programme (NAMP) and in 2003 it became the National Vector Borne Disease Control Programme (NVBDCP) (90). In 2010, 1.4 million malaria cases were reported by routine surveillance of which half were due to *P. falciparum* (91).

GAMETOCYTES IN INDIA

History

Much of the early literature on gametocytemia, and indeed much of all malaria research itself, from India. In part four of a series of paper on malaria treatment, Major JA Sinton of the Indian Medical Service and the director of the Malaria Survey of India, draws the following conclusions in "The occurrence of sexual forms of Plasmodium falciparum in the peripheral circulation" (92):

1) The occurrence of crescents in the peripheral blood of cases of malignant tertian malaria seems to have seasonal periodicity.

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2) The recorded variations in the incidence of crescent carriers in different localities are influenced by various factors.

3) The development of crescents appears to be associated with a lowered immunity of the body.

4) The duration of the life of a crescent may be as long as 40 to 50 days but the great majority disappears within three weeks after the asexual cycle has been destroyed.

5) The reduction of crescent carriers by efficient treatment and the prevention of such carriers conveying the infection to mosquitoes are important factors in all anti-malaria campaigns.

Most of his observations, and the work of that time, remains relevant today and their work would help establish the primary role of the 8-aminoquinoline compounds in gametocytocidal therapy.

Epidemiology

A Medline search of "gametocyte India" (Oct, 2011) returned 20 results of which 7 articles were relevant. Of these four studies (Table 2.1 A) provided limited useful information with most suffering from problems of small samples, non-specific goals, being outdated, and limited area coverage (93–96). Additionally, diagnosis of gametocytes was not validated and studies did not specify the laboratory protocol for microscopy used to assess gametocytemia. Most importantly, none of the studies attempted to characterize individuals who have gametocytes by examining key covariates including age, gender, asexual parasite density, etc. The remaining studies were clinical trials of gametocytocidal agents (primaquine, bulaquine and alpha-beta arteether) (97–99) (Table 2.1 B). Thus, current

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knowledge regarding the distribution and determinants of gametocytes in India is quite

limited.

Treatment

Before the switch to ACT, the use of primaquine for gametocytocidal effect was part

of the national drug policy for *P. falciparum* treatment in India (0.75mg/kg, usual adult dose

of 45mg) (100). Notably, serious side-effects to single dose primaquine remain unreported

after decades of use in millions of patients though this matter has not been the focus of

careful study and pharmacovigilance is poor (74). Recent studies have also challenged this

picture (71). Given the vulnerable nature of malaria endemic populations, Baird and

Surjadjaja have argued for a higher bar of demonstrating safety to ethically use primaquine

noting the current evidence is deficient by any modern medical standard let alone sufficient

to achieve the approval of drug regulatory agencies today (74).

With the addition of ACTs to the drug schedule in 2005 (101), as a second-line

treatment for chloroquine resistant areas, primaquine's role was uncertain as artemisinin acts

against immature gametocytes but does not eliminate transmission (51,58,59,61,62,102–105).

Given that World Health Organization recommendations were ambivalent ("primaquine may

be added" in low transmission areas) in 2006 (106), Indian policy did not specify single dose

primaquine use or disuse (107). In the late 2010 revision of the drug policy (108), when ACT

was extended as the universal first-line treatment for *P. falciparum* across India, primaquine

use was recommended on the second day of ACT treatment given the known, independent

effects of each (primaquine's effectiveness and artemisinin's ineffectiveness on mature

gametocytes) (109), recent evidence from other countries, and stronger WHO language for

low transmission areas (106). Yet, no studies have been conducted in India and evidence of its implementation, in both public and private facilities is lacking (110).

SUMMARY

Knowledge about the epidemiology of gametocytes and efficacy of gametocytocidal treatment is important, yet they remain largely unknown in India, which is the highest burden country outside sub-Saharan Africa. The transmission setting in India is quite different from that of sub-Saharan Africa and local data is needed. Low natural immunity, more adult malaria, generally better access to drugs, and mixed malaria species (111) all affect the risk of gametocytemia and thus we could expect a different epidemiological scenario than described elsewhere. All previous trials of primaquine were conducted in high transmission areas with greater immunity and most of these areas were also primaquine "naïve" (47,70,71,75,112). In India, where primaquine has been used for decades, with one study suggesting the gametocytocidal effect of a single dose has been reduced (97) so results here may not mirror results elsewhere. In addition to the direction of the effect, estimating the magnitude of primaquine's effect in India is important for local cost-benefit analysis needed for policy. Finally, local data of benefit may improve confidence and compliance with primaquine treatment or data suggesting little effect due to India's differences could indicate the need to alter the policy.

FIGURE 2.1. The life cycle of malaria parasites (2)
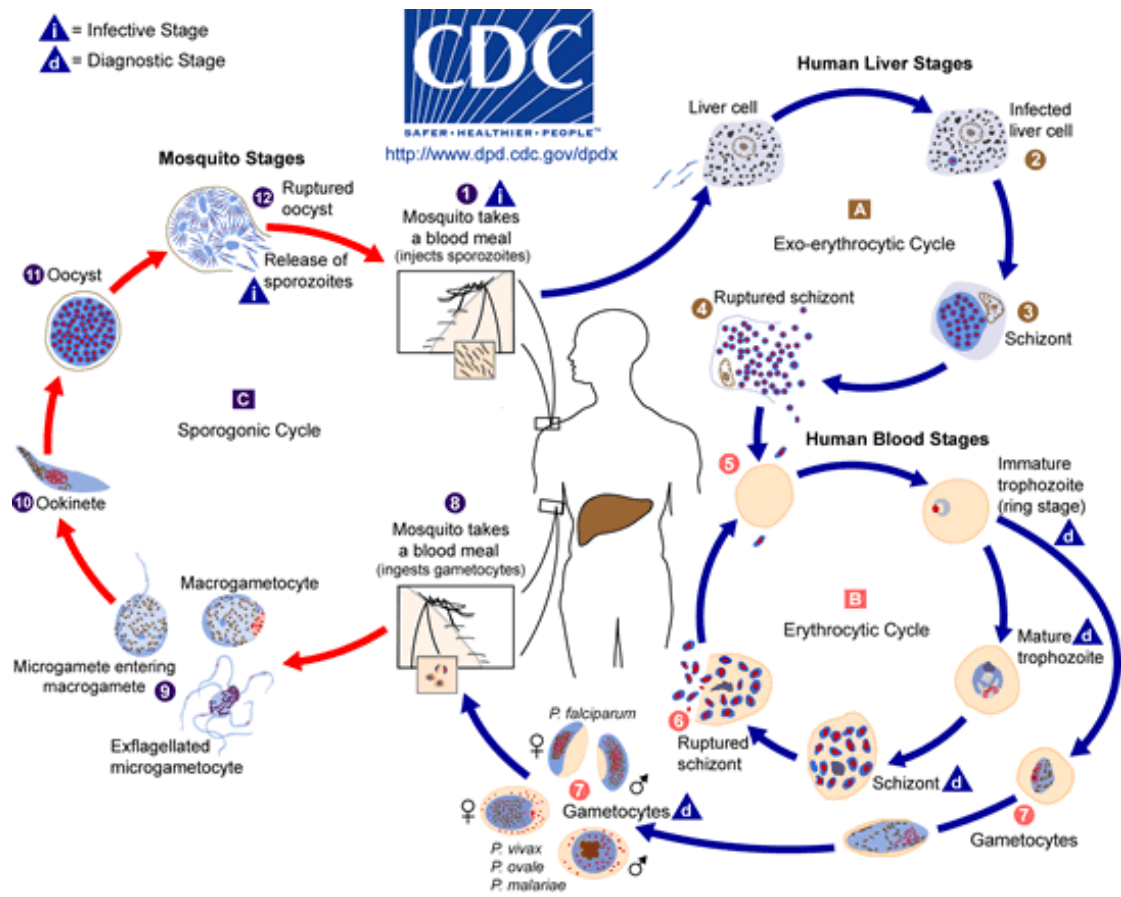
FIGURE 2.2. The timing and duration of *P. falciparum* gametocytemia in relation to the host life-cycle: A. Natural history. B. Treated infection.

A.
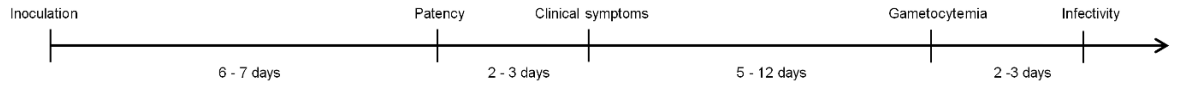
B.
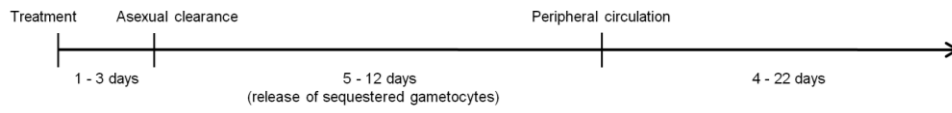
FIGURE 2.3. Malaria endemicity in India by spleen rate, 1941 (79)

Map 21.—Malaria in India.
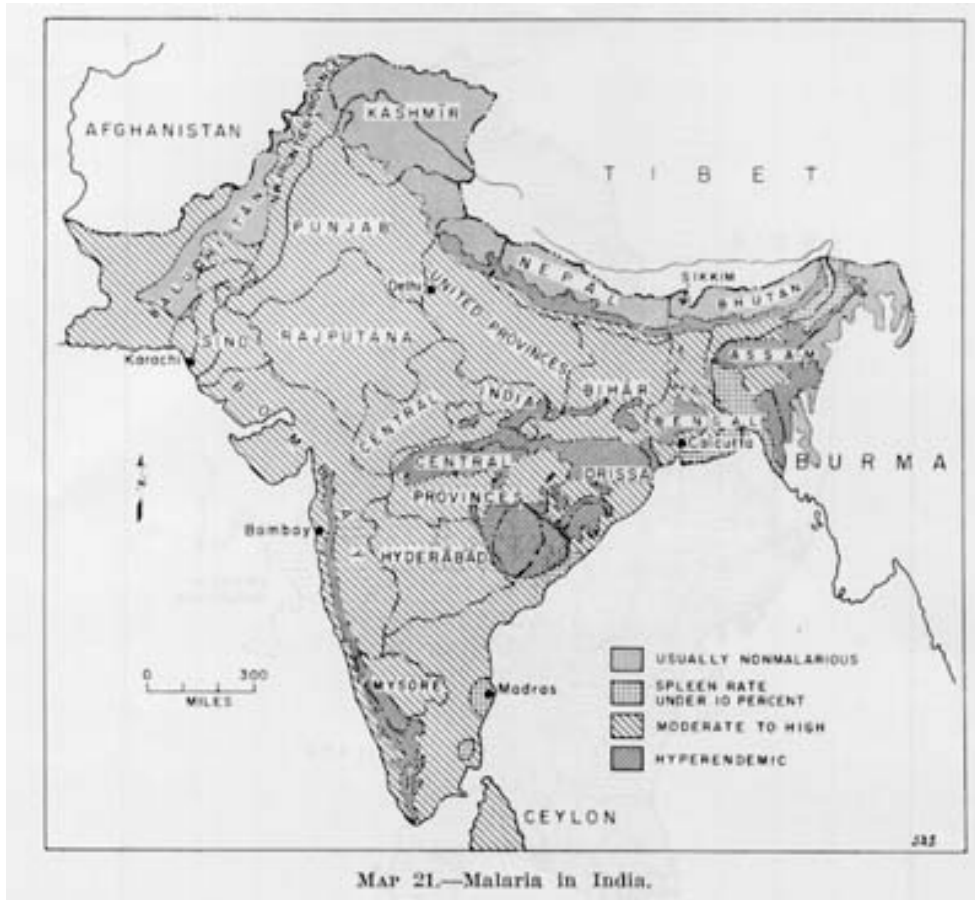
FIGURE 2.4. Reported malaria incidence and P. falciparum proportion in India by NVBDCP 1961-2009 (91)
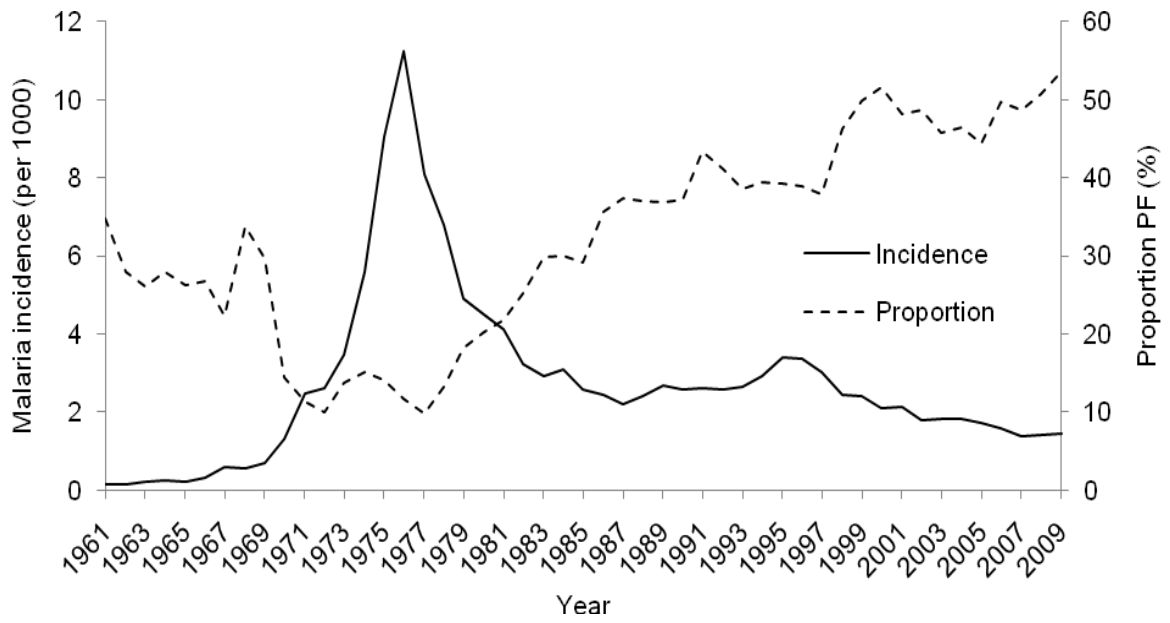
TABLE 2.1. Summary of table of previous published studies on: A. Gametocyte epidemiology in India. B. Trials of primaquine as a gametocytocidal agent in India.

A.

Reference	District, State	Sample size	Gametocytes	Key Results
Rajagopalan et al., 1990 (95)	Koraput, Odisha	714	127	Point prevalence
Mohapatra et al., 1992 (94)	Koraput, Odisha	22	22	Circulation time estimates
Mohapatra et al., 1998 (96)	Nalbari, Assam	--	--	High occurrence in children
Kar et al., 2009 (93)	Laskar, Uttaranchal	--	568	Epidemic and resistance marker

B.

Reference	District, State	Comparator	First-line	Key Results
Gogtay et al., 1999 (113)	Mumbai, Maharashtra	None	CQ	4/15 CQ sensitive infections remained gametocytemic on day 28
Kamtekar et al., 2004 (97)	Mumbai, Maharashtra	No primaquine	Quinine	4% (PQ) gametocytemic day 28 vs 27%
Gogtay et al., 2006 (98)	Mumbai, Maharashtra	Bulaquine	CQ	Clearance of gametocytes by day 28, faster in BQ

Abbreviations: BQ, bulaquine; CQ, chloroquine; PQ, primaquine

CHAPTER THREE: DESCRIPTION OF DATA SOURCES

STUDY DESIGN

The study used existing data from the National Antimalarial Drug Resistance Monitoring System which conducted 22 *P. falciparum* efficacy trials in different sites across India in 2009 and 2010 (Appendix A) (114). However, the use of primaquine as an adjunct therapy was not specified leading to 9 trial sites using it, while 13 sites did not.

Aim 1 used a cross section of the trial population, consisting of data available during the treatment phase (day zero to day two). Aim 2 was a cohort study of 28 days of follow-up among all patients from the efficacy trials who received the full course of treatment. Our study design provided a large sample size from multiple settings across India which assisted with the generalizablity of the findings. Additionally, since we used data from a trial setting using expert staff, the data quality was high.

An alternative study design for Aim 1 could have been a prospective study of an at-risk cohort to examine the incidence and changes in gametocytemia over time. An alternative study design for Aim 2 could have been a randomized controlled trial to determine the efficacy of primaquine. However, both designs would have been limited in scale or too expensive and difficult to conduct over multiple geographic areas. By using existing data, this study was practical, feasible, and inexpensive to conduct.

STUDY SITES

A total of 11 states were selected: Andhra Pradesh, Assam, Chhattisgarh, Gujarat, Jharkhand, Madhya Pradesh, Maharashtra, Meghalaya, Odisha, Rajasthan, and West Bengal were selected. Among these states 21 districts were selected: Angul, Baran, Betul, Bilaspur, Chhindwara, Dhenkanal, Dumka, Jalpaiguri, Kanker, Kolkata, Latehar, Mumbai, Nagaon, Pratapgarh, Ranchi, Sambalpur, Silvasa, Simdega, Surat, Vishakhapatnam, and West Garo Hills (Figure 3.1). We could not obtain data from one site (Gadcharoli) because gametocytemia was not recorded in case record forms and slides could not be re-examined. NVBDCP and NIMR purposively selected the study areas to represent *P. falciparum* transmission settings across the country. Geographic clusters were associated with different malaria ecotypes: Western India as Gujarat, Mumbai, and Rajasthan, Central India as Andhra Pradesh, Chhattisgarh, Gadcharoli, Jharkhand, Madhya Pradesh, and Orissa, and Eastern India as the Assam, Meghalaya, and West Bengal sites (85,115). While the ecotype of malaria within clusters may vary, for example urban Western India compared to rural Western India, finer resolution was not possible with the available information. The ecotypes of the clusters was as follows: Western – unstable rural or urban transmission driven by *Anopheles culicifacies* and *A. stephensi*, Central – stable forest-related transmission driven by *A. fluviatilis*, and Northeastern – stable de-forestation and forest-related transmission driven by *A. minimus* and *A. dirus* (116).

STUDY POPULATION

The source population was the entire population enrolled in the trials during the study period (2009-2010). The requirements for inclusion included *P. falciparum* monoinfection, any age, asexual density greater than 500/uL and less than 100,000/uL, without signs of

severe malaria, non-pregnant, and willing to consent to follow-up. Some patients ineligible on parasite density (either wrong species, out of range, only rapid test used, etc) were enrolled, but withdrawn from the study at a later date. Patients were enrolled through routine fever screening at clinics or through active case detection in the surrounding community by the study team. The main advantages of the study population were its size, representativeness of different malaria transmission settings and ecotypes, and quality of monitoring.

DATA COLLECTION

Data were collected as previously described (114). Each trial had 28 days of follow-up with patient contact during the first three days of treatment and then at one week intervals. Thus the follow-up schedule consisted of the following days: 0, 1, 2, 3, 7, 14, 21, and 28. Patients were also advised to return at any time if they had a fever or otherwise felt unwell. Demographic information was collected at enrollment and physical examination and thick and thin blood smears were performed at each study visit. Patients were treated on days zero to two with AS+SP (4mg/kg for three days plus 25/1.25mg/kg) using age-wise dosing as per national guidelines with or without primaquine on the third day of treatment (0.75mg/kg, usual adult dose of 45mg). Teams received standardized trainings and all studies were supervised under one principal investigator using a common protocol (117) and there was central quality control of the data management as well as outcome classification. Data were collected in a standardized case record form (Appendix B) and then double entered into the standard World Health Organization 28 day therapeutic efficacy excel format.

DATA MANAGEMENT

Existing therapeutic efficacy data was consolidated into a single database from its previous composition of multiple Microsoft Excel workbooks. We incorporated range checks to investigate plausible values for each data field. The cleaned data was imported electronically into STATA (v10).

FIGURE 3.1. Map of India with *P. falciparum* trial districts (in red), and *P. vivax* trial districts (in blue) conducted through the National Antimalarial Drug Resistance Monitoring System, 2009-2010
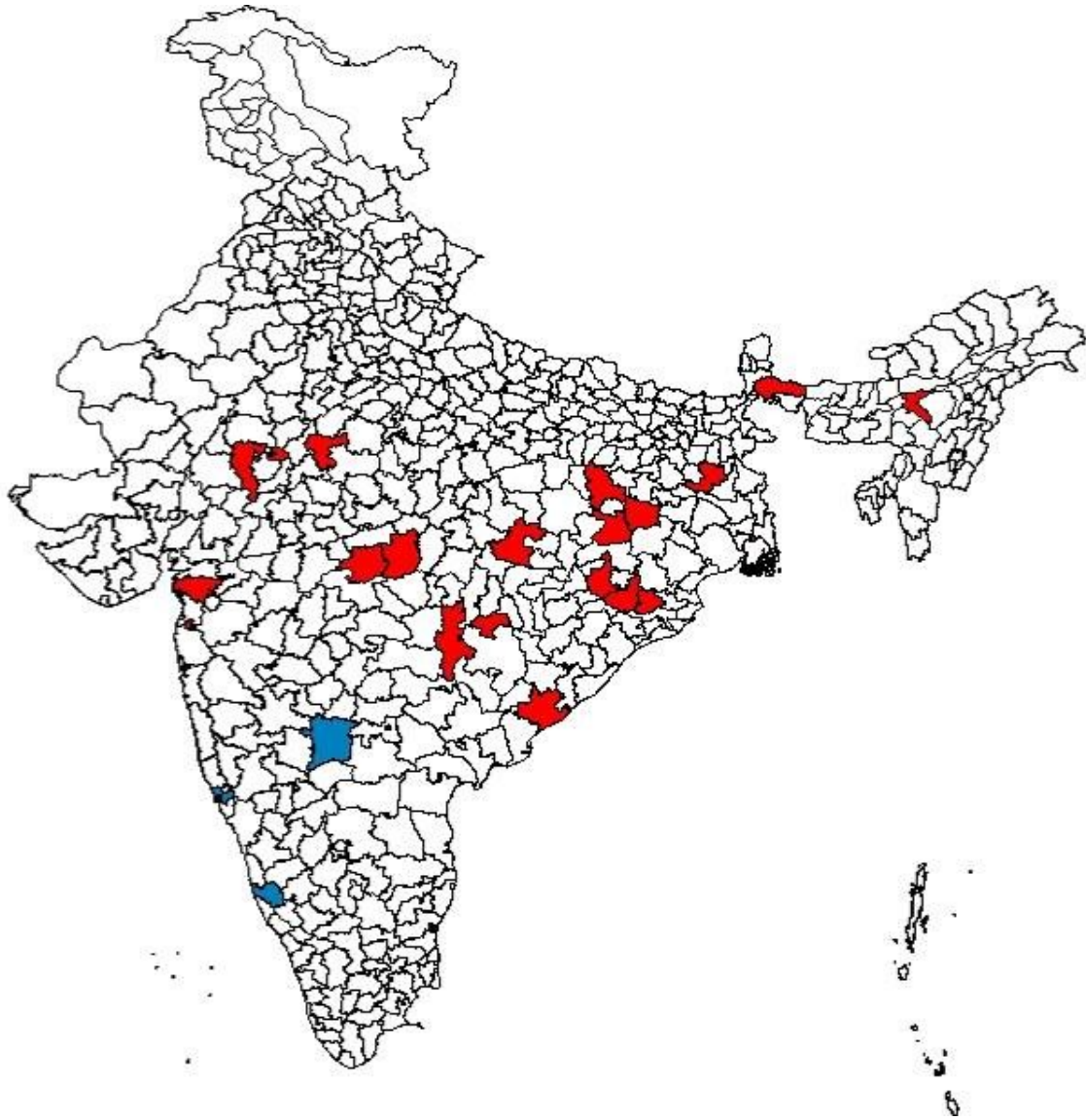
CHAPTER FOUR: METHODS

SPECIFIC AIM 1

Exposure and outcome assessment

Risk factors for gametocytemia that would be easily identifiable in field settings to ensure programmatic relevance were the exposures of interest: age, region, gender, previous antimalarial drug intake, current fever, history of fever, and asexual parasite density. Exposures were assessed record review of surveyed demographic or clinical information except for current fever used a digital thermometer to take the axillary temperature and asexual parasitemia which was assessed by microscopy as described below. The outcomes of interest were gametocytemia and gametocyte density.

Diagnosis of gametocytes was by light microscopy. Briefly, routine oil immersion reading at 1,000X on Giemsa stained thick smears was used. Slides were counted until 200 white blood cells (WBC) if gametocytes were detected by then or until 1,000 WBCs to declare a slide negative. We assumed 8,000 WBC per µL of blood to obtain a final density per microliter of blood. All treatment failures and 10% of slides were randomly cross-checked during the initial study for quality control purposes. Additionally, we cross-checked the entire follow-up series from ten patients with day zero gametocytemia selected at random and ten patients without day zero gametocytemia selected at random to specifically determine the validity of gametocyte presence. Recorded data was 96% sensitive and 100% specific relative to expert re-assessment suggesting the data was appropriate for use.

Case and covariate definitions

We defined gametocytemia as the presence of gametocytes, in any day between enrollment and day two of follow-up, in a patient eligible for the therapeutic efficacy study in 2009 or 2010. The half-life of mature gametocytes is estimated at four to six days once in peripheral blood (13). Thus, patients with gametocytemia on the first day of follow-up may have been gametocytemic at enrollment. At the least, they would have benefited from a gametocytocidal intervention. Gametocytemia detected later in follow-up however, may have different origins and may not benefit from a gametocytocidal intervention given during treatment. For example, gametocytogenesis from persisting asexual stages, re-infection, or the release of sequestered developing gametocytes could explain any later onset gametocytemia.

We categorized age using cutoffs used in age-wise treatment blister packs (<1, 1-4, 5-9, 10-14, and ≥15 years categories) and asexual parasite density using cut-offs used in previous studies (<5,000, 5,000-9,999, 10,000-49,999, and ≥50,000 parasites/μL) (Table 4.1). The recall period for history of fever was the past 48 hours and for taking an antimalarial drug was the past one week. We defined current fever as an axillary temperature ≥37.5°C at the time of enrollment. We designated area using geographic clusters associated with different malaria ecotypes: Western India as Gujarat, Mumbai, and Rajasthan, Central India as Andhra Pradesh, Chhattisgarh, Gadcharoli, Jharkhand, Madhya Pradesh, and Orissa, and Northeast India as the Assam, Meghalaya, and West Bengal sites (77,78). While the ecotype of malaria within clusters may vary, for example urban Western India compared to rural Western India, finer resolution was not possible without a loss of power.

Complete case analysis

We conducted a complete case analysis using all patients completing two follow-up visits after enrollment. None of the predictors had missing data. Missing gametocytemia data, due to withdrawal and loss to follow-up during the three-day treatment phase, was less than 3% of the overall sample. We made the assumption that missing data on gametocytemia was missing at random within the levels of covariates. Given these assumptions complete case analysis will lead to some loss of power but not appreciable bias. The missing data is less than 5% of the overall sample suggesting such censoring is unlikely to be informative.

Data analysis

To describe distribution of gametocytes in the population, we calculated the prevalence of gametocytes among levels of predictors and the overall population. We collapsed categories of continuous variables where the prevalence of gametocytemia was similar among adjoining groups. In bivariate analyses these characteristics were compared among patients with and without gametocytemia using the Chi-squared, Fishers exact, t-test or Wilcoxon-rank sum as appropriate for inclusion in the logistic regression model. We also calculated the proportion of parasites that were gametocytes by dividing the enrollment gametocytemia by the sum of the enrollment gametocyte and asexual parasite densities. We multiplied the mean geometric density of the maximum or the mean gametocytemia during day zero through two and the number of gametocytemic individuals in each age category to estimate the gametocyte population. We calculated the proportion of the potential reservoir for transmission in each age category by dividing the dividing the gametocyte population of that age category by the total gametocyte population.

We used unconditional multivariate logistic regression to build a reference predictive model between the demographic and clinical factors and gametocytemia. To account for the clustering of data in each trial, we used cluster robust error with district as the unit. We estimated the crude prevalence odds ratio and its 95% confidence interval between predictors and gametocytemia. We included all predictors associated with gametocytemia (p<0.25) in the bivariate analysis. We assessed collinearity between each pair of predictive factors and selected among collinear variables based on their operational utility. We evaluated two-way interactions of each effect measure and retained all product terms with p values <0.25. To simplify field use of the algorithm, we examined reduced models which had adequate fit and similar predictive power to the reference model. We used backwards elimination using the Wald test to remove predictors with p<0.10 starting with interaction terms and proceeding to the variable with the highest p value. We assessed changes in the performance of the reduced model due to removing variables or collapsing across categories using the area under the receiver operating characteristic (ROC) curve. We evaluated model fit using the Hosmer-Lemeshow test.

We then created a scoring system from the logistic model output using the regression coefficient, to preserve the multiplicative nature of the score, of each predictor in the reduced model. We multiplied the regression coefficient by ten and rounded to the nearest integer to simplify score use (118). A final score for each patient was obtained by summing the individual scores from their predictor values. To determine the utility of the scoring system, we evaluated the sensitivity, specificity, false negatives, false positives and the area under the ROC curve of different score cut-points. We calculated false negatives using the formula (1 − sensitivity) * gametocytemia prevalence * N and false positives as (1 − specificity) * (1 − gametocytemia prevalence) * N. We also calculated the percent of the population correctly

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classified and the percent of the population which would be treated if scores were used to target gametocytocidal therapy. False positive and false negatives were not formally weighted but considerations around each will be discussed in light of program goals (control or elimination).

Power

Assuming a control:case ratio of 9:1 we have more than 80% power to detect any risk factors with an POR of 2 and a prevalence among control of 7.5% or greater (Figure 4.2 A). Multivariate analysis may decrease power but using these conservative assumptions, this study has sufficient power to accomplish its aims.

Ethical clearance

The Scientific Advisory Committee of the National Institute of Malaria Research, and the Institutional Review Board of the University of North Carolina approved the secondary analysis.

SPECIFIC AIM 2

Exposure and outcome assessment

Primaquine therapy was the main exposure. We obtained primaquine use information from the site physician. While site wise practices differed, physicians indicated they administered primaquine uniformly, i.e. to all patients or none on the same day and at the standard dose, within sites so group exposure was used. The main outcome was gametocytemia and secondary outcomes were gametocyte density and reported adverse events. Gametocyte assessment was conducted as described in Aim 1. Adverse events were

assessed through history taking at each study follow-up visit through patient complaint or recall.

Case and covariate definitions

We defined pre-treatment gametocytemia as the presence of gametocytes on or before day two in a patient a patient eligible for the therapeutic efficacy study in 2009 or 2010.We defined post-treatment gametocyte clearance as the first day without gametocytemia after day two in a patient with pre-treatment gametocytemia. We defined an event of post-treatment gametocytemia as the presence of gametocytes on any follow-up day after day two in a patient eligible for the therapeutic efficacy study in 2009 or 2010. We excluded gametocytemia at the time of failure for patients with recrudescence or re-infection.

For measuring exposure time in days we censored at the midpoint of the interval and for measuring exposure time in weeks we censored at the end of the interval. We reported the week time data to enable comparison with previous literature. For the measurement of post-treatment gametocyte circulation, patients who completed follow-up accrued an additional week of exposure in order to incorporate the contribution of individuals who remained positive at the end of day 28. Thus, the total post-treatment exposure time of 32 days is congruent with biological estimates of the maximum circulation time of gametocytes (13). We used the covariates and coding strategies described in Aim 1. (Table 4.1)

Causal diagram

We constructed a causal diagram of the effect of primaquine on gametocytemia using substantive knowledge from literature and experience. We used the DAG program (http://epi.dife.de/dag/) to conduct path analysis to determine the minimally sufficient and

other sufficient adjustment sets (in which case the adjustment is unbiased but precision is lost compared to minimally sufficient adjustment if the diagram is correctly specified and the other sets are correctly measured).

Complete case analysis

We conducted a complete case analysis using all patients who completed treatment. None of the predictors had missing data. Missing gametocytemia data, due to withdrawal and loss to follow-up during the treatment phase, was less than 3% of the overall sample. We made the assumption that missing data on gametocytemia was missing at random within the levels of covariates.. Given these assumptions complete case analysis will lead to some loss of power but not appreciable bias. The missing data is less than 5% of the overall sample suggesting such censoring is unlikely to be informative.

Data analysis

We tabulated the clinical and demographic characteristics, as well as the primary and secondary study outcomes, of patients who received and did not receive primaquine. We collapsed categories of continuous variables where the prevalence of gametocytemia was similar among adjoining groups. We calculated the mean and median gametocyte clearance times by treatment group and compared them using the t-test and ranksum tests. We calculated the incidence, incidence rate ratios, and incidence rate differences over follow-up time among the primaquine and no primaquine groups.

We modeled the effect of primaquine by estimating the post-treatment clearance rates of gametocytes among patients with gametocytes at enrollment and by comparing the circulation of post-treatment gametocyte-weeks among the entire population. We estimated

crude and adjusted relative measures of effect, hazard ratios and incidence rate ratios respectively, as these were readily interpretable and generalizable across our diverse study sites. We used Cox regression with the Efron method for ties (119) with clustered (on site) robust standard error and Poisson regression using general estimating equations with unstructured correlation matrixes and robust clustered (on patient) standard error respectively.

Based on our diagram, adjustment on district was sufficient to control confounding bias. However, we controlled for region instead since the exposure, primaquine, did not vary by district. To assess the robustness of the measure of effect to uncontrolled confounding, we also produced estimates adjusted for age and parasite density, variables strongly associated with gametocytemia, as well as adjusted for all covariates whose distribution differed between the two arms in bivariate analysis using t-tests, chi-square, or fisher's exact test as appropriate (p value<0.10). We included an interaction term between region and primaquine and assessed effect measure modification if the Wald test for the interaction term was p<0.2. We used a backwards elimination strategy to retain confounders which changed the estimate by at least 10%. In the Cox model we compared the model with and without discrete time (marginal) due to the large number of tied events and assessed the proportional hazards assumption by examining the -log(log(survival)) over time for parallel trends between the groups (Table 4.3). We compared the Poisson model to a negative binomial model, using a generalized linear model, using a likelihood ratio test with criteria p<0.05 to ensure appropriate fit with outcome dispersion.

The time at risk was measured in intervals and not continuously, i.e. on day 7, day 14, etc. A patient gametocytemic at day 7 and not gametocytemic on day 14 may have cleared gametocytemia at any point during the interval which potentially misclassifies the exposure

time. We used the same time within the interval to censor for both groups which assumed no difference in inter-interval clearance and could underestimate the measure of effect. The inter-interval time likely differs by primaquine status since the drug accelerates gametocyte clearance. Based on previous studies, we conducted a sensitivity analysis assuming 50% reduction in time at risk in a given interval among primaquine exposed patients. We plotted the mean area under the curve (AUC) for gametocyte density for each group by study visit. We excluded outliers of high pre-treatment gametocytemia (maximum density >2,000/μL, n=5) which disproportionally influenced the AUC. We calculated the AUC per day using the formula of Mendez et al. with the addition of the area for gametocytemia remaining at the end of follow-up (120). We compared the median AUC per day for each arm using the ranksum test and described differences using percentile cut-offs.

Sensitivity analysis

The time at risk was measured in intervals, i.e. Day 7, Day 14, and so forth. A person who was gametocytemic on Day 7 and not gametocytemic on Day 14 could have changed status on Day 8 or Day 13 – a large difference which potentially misclassifies the exposure time and is likely to differ by primaquine status. The use of the mid-point of the interval, which will be our primary method, could underestimate the measure of effect as it assumes no benefit for inter-interval clearance among a primaquine exposed individual which is unlikely. Thus, we will conduct a better case sensitivity analysis of the measure of effect using an alternate times at risk where all those primaquine exposed experienced clearance 50% quicker within the interval compared to those who did not receive primaquine. This value is similar to previously measured effect estimates of primaquine given with an ACT (47,75,76).

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The no primaquine to primaquine ratio is approximately 2:1. The prevalence of gametocytemia among the exposed is assumed at 5% (approximately the day 28 prevalence in the primaquine arm) and we have more than 90% power to detect an IRR >2 (Figure 4.2 B). With our sample size of 156 we have more than 90% power to detect a HR of 0.5 assuming 10% withdrawal and 80% of patients failing during the follow-up period, and nearly 80% power to detect a HR of 0.6. (Figure 4.2 C). Multivariate analysis may slightly decrease power but using these conservative assumptions, this study has sufficient power to accomplish its aims.

The Ministry of Health and Family Welfare, the Scientific Advisory Committee of the National Institute of Malaria Research approved the trials and the Institutional Review Board of the University of North Carolina approved the secondary analysis.

LIMITATIONS

Our methods have several limitations. First, the sensitivity and specificity of microscopy, could affect the results. *P. falciparum* gametocytes are very distinct (crescent forms) and specificity of microscopy is thus high. However, because gametocytes are low-density, microscopy which has a limit of 8 parasites per μL (when 1,000 WBCs are counted) may miss positive samples near or below that threshold (45). The sensitivity would also be differential in respect to exposures that modify density, such as primaquine use. However, because the density of gametocytes is essential to its mechanism, i.e. the probability of infection of mosquitoes is positively correlated with gametocyte density, microscopy may provide a useful cut-off of functionality. Stated differently, a gametocytemia of 1 per μL is not equivalent to a higher gametocytemia as it is functionally less infective (52). Thus, the

sensitivity of microscopy could be considered adequate as well as non-differential in respect to functional gametocytemia. Similar estimates of the effect of primaquine, in one study which used two outcomes – binary microscopic gametocytemia or area under the curve for molecular gametocytemia – suggests that bias due to outcome misclassification may be minimal (47,70). Other studies which sought to describe the association of gametocytemia with age and as a portion of the total parasite population by QT-NASBA and by microscopy or estimate the circulation times of gametocytes demonstrated no difference in trends by technique (13,121).

Second, we completed enrollment at each site over one to two months preventing the analysis of seasonal trends for gametocytemia.

Third, our population cross-section is not representative of the population at-risk. It represented individuals attending the public sector health clinic for their fever treatment or those in communities which were accessible to active case detection. However, it is representative of the population encountered by the national control programme through their active and passive case detection. This also means that all individuals were symptomatic. We did not assess the contribution of asymptomatic carriers to transmission but, at present, there may not be valid means to do so (122). The population thus fitted our goal - to observe the population amenable to current and future interventions through the control programme.

Fourth, primaquine use was not randomized. While we controlled for several sets of covariates and assessed effect measure modification, confounding due to unmeasured causes may bias our estimated effect and the possibility of heterogeneity of the effect cannot be excluded with the available power (123).

Finally, we used the presence of gametocytes as a proxy for infectiousness. Infectivity is modified by a number of factors and its direct assessment through membrane-feeding

experiments, which are labour and time intensive, would have precluded the type of large

survey needed for generalizable results (124,125).

TABLE 4.1. Potential variables, coding structure, and notes for use in data analysis

Variable	Coding	Notes
Gametocytemia	0=No 1=Yes	
Day of classification	3.5 10.5 17.5 24.5 31.5	Continuous variable but measured at intervals. Censoring at mid-point of the week
Week of classification	4/7 1	Continuous variable but measured at intervals. Censoring at end of the week
Gametocyte density(/μL)	Continuous	
Primaquine	0=No 1=Yes	External variable, site-wise exposure
Sex	0=Female 1=Male	
Antimalarial drug intake in the past week	0=No 1=Unknown/Yes	Less assumptions than listing unknown as missing
Current fever (axillary temp ≥37.5°C)	0=No 1=Yes	
History of fever in 24 hrs	0=Yes 1=No	
Asexual parasite density (/μL)	0=<1000 1=1000-5000 2=5000-50000 3=≥50000	Continuous variable, cut-offs similar to traditional semi-quantitative grading (+ - ++++), evaluated using indicator variables

Variable	Coding	Notes
Age (years)	0=0-4 1=5-9 2=10-14 3=15-49 4=≥50	Continuous variable, cut-offs used age-blister packs and correspond to malaria immunity / exposure risks, evaluated using indicator variables
Classification	0=Withdrawal 1=Lost to follow-up 2=ACPR 3=ETF 4=LCF 5=LPF	Categorical based on WHO protocol, evaluated using indicator variables
District	22 districts	Categorical variable, evaluated using indicator variables
Region	0=Western 1=Central 2=Northeast	Categorical based on ecotype, evaluated using indicator variables

FIGURE 4.1. Causal diagram of the relationship between primaquine use and post-treatment gametocytemia
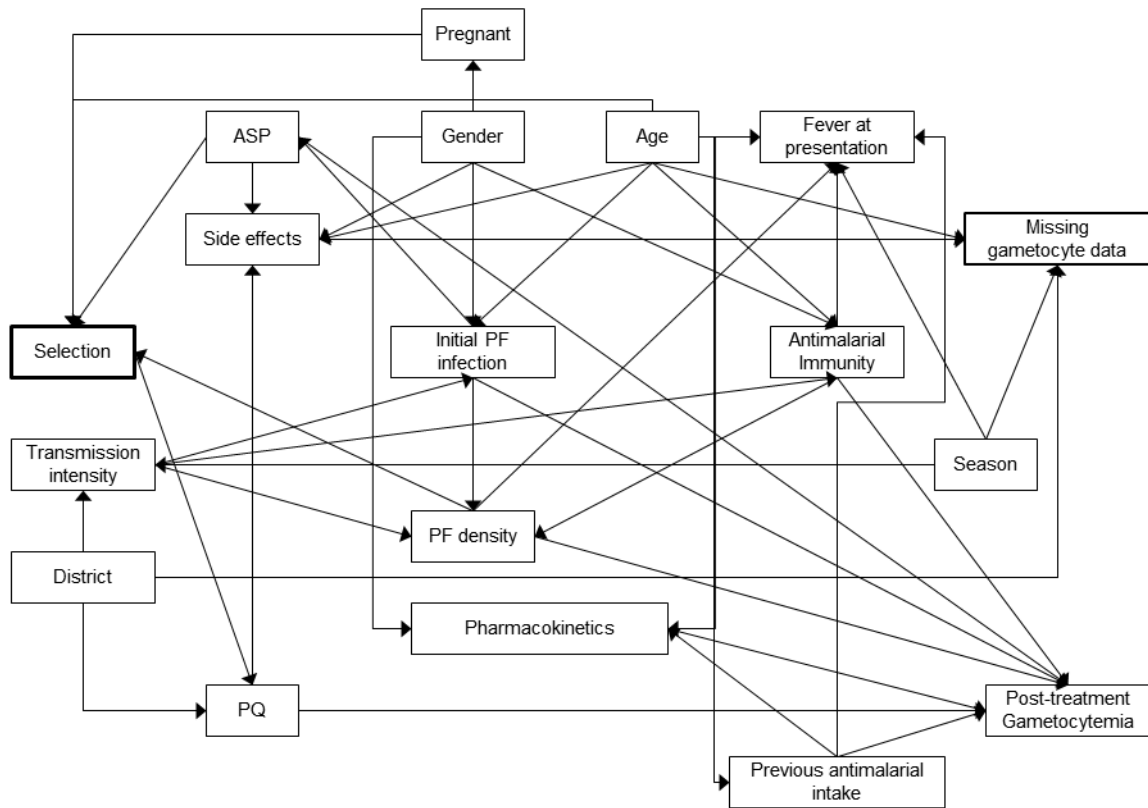
FIGURE 4.2. Power available for detecting associations with or effects on gametocytemia. A. Odds ratio (lines), different exposure prevalence, and power. B. Incidence rate ratios, given assumptions, and power. C. Hazard ratio, given assumptions, and power

A.

B.
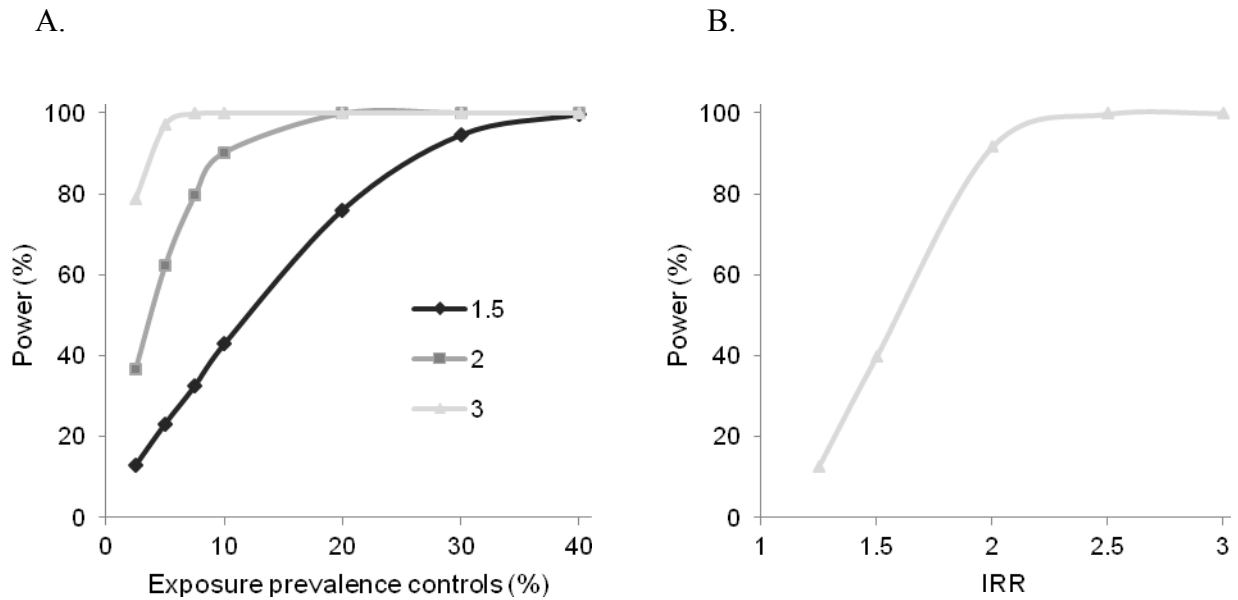
C.

Power	N	E	HR	SD	Alpha*	Pr(E)	W
.8	9	6	.1	.5	.05	.8	.1
.9	12	8	.1	.5	.05	.8	.1
.8	17	13	.2	.5	.05	.8	.1
.9	23	17	.2	.5	.05	.8	.1
.8	31	22	.3	.5	.05	.8	.1
.9	41	29	.3	.5	.05	.8	.1
.8	52	38	.4	.5	.05	.8	.1
.9	70	51	.4	.5	.05	.8	.1
.8	91	66	.5	.5	.05	.8	.1
.9	122	88	.5	.5	.05	.8	.1
.8	168	121	.6	.5	.05	.8	.1
.9	224	162	.6	.5	.05	.8	.1
.8	343	247	.7	.5	.05	.8	.1
.9	459	331	.7	.5	.05	.8	.1

* two sided

FIGURE 4.3. Tests to ensure appropriate fit of the Cox proportion hazards model. A. Proportional hazards assumptions. B. Comparison with Kaplan-Meir function.

A.
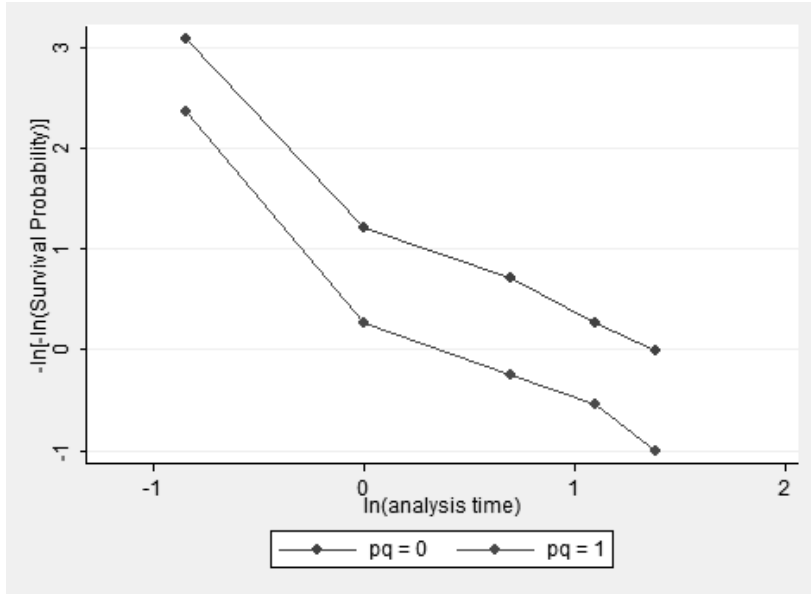
B.
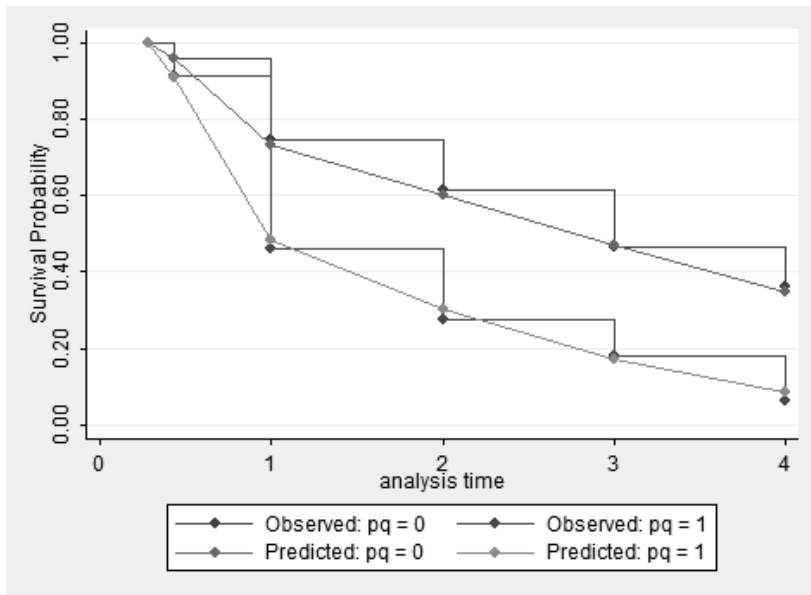
**CHAPTER FIVE: THE EPIDEMIOLOGY OF *PLASMODIUM FALCIPARUM*
GAMETOCYTEMIA IN INDIA: PREVALENCE, AGE-STRUCTURE, RISK
FACTORS AND THE ROLE OF A PREDICTIVE SCORE FOR DETECTION**

INTRODUCTION

Gametocytes are the sexual stage of *Plasmodia* life cycle that render malaria patients

infectious to mosquitoes and propagate transmission. The proportion of patients infected by

P. falciparum with gametocytemia and the duration and density of that gametocytemia vary.

Although gametocytes are central to understanding transmission, few studies have tried to

demarcate the infectious reservoir (15). This may be because the infectious reservoir is not an

important determinant of the intensity of transmission relative to high vectorial capacity in

high transmission areas (17). However, in low and moderate transmission areas, the

proportion of infectious hosts is critical to the maintenance of endemicity (17). Interventions

for detecting and treating gametocytemia also differ from those used for asexual parasitemia.

So an improved understanding of the epidemiology of gametocytemia opens the possibility

of distinct transmission-blocking control strategies. With recent reductions in malaria

transmission in many countries and increased focus on elimination worldwide, interest in

gametocytemia is gaining traction.

The detection of gametocytes is complicated by their usual low density in peripheral

blood. Gametocyte density is generally lower than that of asexual parasites (typically less

than 5% of the total parasite population) and levels below the detection limit of microscopy

can infect mosquitoes (23). The low density of gametocytes coupled with inadequate

laboratory standards and the heavy workloads of technicians makes the diagnosis of gametocytemia difficult in routine settings. Recently, molecular methods that detect gametocyte-stage specific RNA transcripts have been employed in research (43–45) but these may not be feasible for routine public health use. Associations of gametocytemia with easily discerned factors including age, season, and symptoms such as fever at the time of presentation could provide an alternative strategy for targeting gametocytocidal interventions. Clinical algorithms for predicting gametocytemia among diagnosed malaria patients could help improve its detection.

The control of malaria is a key challenge for India which reported 1.5 million cases in 2010 (126). The reduction of transmission is a priority; most of the country consists of low and moderate malaria endemicity. The transmission of malaria in India is arguably the most complex in the world given the large geographic area, the presence of both major parasite species, a wide range of ecotypes and vectors, and the enormous population (89). In addition, lower acquired immunity, more adult malaria, better access to drugs, and mixed species infections alter the epidemiology of gametocytemia in India compared to sub-Saharan Africa (111). Pre-independence Sinton and others studied the crescents of malignant tertian malaria primarily with respect to treatment and spleen size (92). Since 1990, only 4 published studies have described gametocytemia in India but these suffer from small sample sizes and limited area coverage (93–96). Most importantly, no study characterized the subpopulation with gametocytemia.

The goal of this study was to describe the epidemiology of *P. falciparum* gametocytemia in India and determine whether a clinical predictive model could improve its detection. First, we collected data from a national network of sentinel sites and validated the diagnosis of gametocytes. Second, we examined the prevalence of gametocytemia in relation

to clinical and demographic factors to understand its distribution among subpopulations of our study. Third, we calculated the contribution of different age groups to the reservoir for potential transmission using gametocyte density. Last, we created predictive scores for gametocytemia and calculated the sensitivity and specificity of different cut-points to determine the utility of an algorithmic approach for detection.

METHODS

Study sites and population

We utilized data from 22 *P. falciparum* therapeutic efficacy trials conducted through the National Antimalarial Drug Resistance Monitoring Network of India in 2009 and 2010 (114). The National Vector Borne Disease Control Programme and the National Institute of Malaria Research purposively selected the study sites to represent *P. falciparum* transmission settings across the country. We could not obtain data from 1 site (Gadcharoli) because gametocytemia was not recorded in case record forms and slides could not be re-examined. We included all patients eligible for the World Health Organization (WHO) therapeutic efficacy trial protocol: patients with *P. falciparum* monoinfection, febrile or with a history of fever, asexual parasite density greater than 500/µL and less than 100,000/µL, and willingness to consent to follow-up (117). We excluded pregnant patients and those with signs of severe malaria. The purpose of these trials was to measure the clinical and parasitological response of malaria patients when treated with first-line drugs used in national policy. The study population represents a cross-section of the patients who presented to the local clinic or were recruited through active case detection in nearby communities.

Data collection

The data collection methods have been previously described (114). Briefly, clinical and demographic information were recorded from each patient at enrollment. Patients were followed during the first 3 days of treatment (artesunate plus sulfadoxine-pyrimethamine, 4mg/kg for three days + 25/1.25mg/kg as per national guidelines) and then at weekly intervals from enrollment until day 28. At each follow-up visit a physical exam was conducted and thick and thin blood smears were prepared. Using routine oil immersion reading at 1,000X on Giemsa-stained thick smears, slides were counted until 200 white blood cells (WBC) if gametocytes and asexual stages were present or until 1,000 WBC to declare a slide negative for both. A count of 8,000 WBC/μL of blood was assumed to obtain a final density for asexual and sexual parasites. The data were double-entered into the WHO therapeutic efficacy database and blood slides were cross-checked by expert microscopists. Existing therapeutic efficacy data was consolidated into a single database after range checks to investigate plausible values for each data field.

Case and predictor definitions

We defined gametocytemia as the presence of gametocytes in the peripheral blood smear, in any visit between day 0 and day 2 of follow-up, in a patient eligible for a therapeutic efficacy study in 2009 or 2010. The half-life of mature gametocytes is estimated at 4 to 6 days once in peripheral blood (13). Thus, patients with gametocytemia on the first day of follow-up may have been gametocytemic at enrollment. At the least, they would have benefited from a gametocytocidal intervention. Gametocytemia detected later in follow-up however, may have different origins and may not benefit from a gametocytocidal intervention given during treatment. For example, gametocytogenesis from persisting asexual

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stages, re-infection, or the release of sequestered developing gametocytes could explain any later onset gametocytemia.

We selected predictors associated with gametocytemia in prior literature that could be feasibly identified during routine curative care: age, sex, region, previous antimalarial drug intake, current fever, history of fever, season, and asexual parasite density (15). We categorized age with cutoffs used in age-wise treatment blister packs (<1, 1-4, 5-9, 10-14, and ≥15 years categories) and asexual parasite density with cut-offs used in previous studies (<5,000, 5,000-9,999, 10,000-49,999, and ≥50,000 parasites/µL). The recall period for history of fever was the past 48 hours and for taking an antimalarial drug was the past week. We defined current fever as an axillary temperature ≥37.5°C at the time of enrollment. We designated region using geographic clusters associated with different malaria ecotypes: western India as Gujarat, Mumbai, and Rajasthan, central India as Andhra Pradesh, Chhattisgarh, Gadcharoli, Jharkhand, Madhya Pradesh, and Orissa, and northeast India as the Assam, Meghalaya, and West Bengal sites (85,115). Multiple ecotypes, each with different implications for both transmission intensity and malaria control strategy, exist within each of these broad regions (127). However, the categorization captures the dominant ecotype, e.g. forest malaria in central India, or agglomerates overlapping ecotypes of not so dissimilar transmission intensity as with rural, agricultural malaria and urban, migrant malaria in western India. Finer resolution was not possible without a loss of power. We classified season by month of enrollment: monsoon – June-August, post-monsoon – September-November, and winter – December and January.

Data analysis

We analyzed all patients who completed 2 follow-up visits after enrollment. None of the predictors, age, sex, region, previous antimalarial drug intake, current fever, history of

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fever, season, and asexual parasite density, had missing data. Missing gametocytemia data, due to withdrawal and loss to follow-up during the three-day treatment phase, was less than 3% of the overall sample. We calculated the prevalence of gametocytemia among levels of predictors and in the overall population. We collapsed categories of age and parasite density where the prevalence of gametocytemia was similar among adjoining groups. We also calculated the proportion of parasites that were gametocytes by dividing the enrollment gametocytemia by the sum of the enrollment gametocyte and asexual parasite densities. To calculate the proportion of the reservoir for potential transmission in each age category we first multiplied the mean geometric density of the maximum or the mean gametocytemia during day 0 through day 2 in the age category by the number of gametocytemic individuals in the age category. Conceptually, the result can be considered the gametocyte load of the age category. Second, we summed the gametocyte load of each age category to obtain the population gametocyte load. Third, we divided the gametocyte load of each age category by the total gametocyte load leading to the proportion of the reservoir for potential transmission represented by the age category. The use of transmission is qualified with the term "potential" as our approach ignores differences in gametocyte infectivity (due to immunity) and vector biting rates (due to uncovered surface area, etc) between the age groups.

We used unconditional multivariate logistic regression to build a reference predictive model between the demographic and clinical factors and gametocytemia. To account for the clustering of data in each trial, we estimated cluster robust standard errors with district as the unit (128). We estimated the crude prevalence odds ratio and its 95% confidence interval between each predictor and gametocytemia. We included all predictors associated with gametocytemia (P value < .25 to avoid the exclusion of important variables) in those bivariate analyses. We assessed collinearity between each pair of predictive factors by

calculating the odds ratio. We selected among collinear variables (odds ratio > 3) based on their substantive value. We evaluated two-way interactions between all pairs of predictors and retained all product terms with P value < .25.

To simplify field use of the algorithm, we examined reduced models that had similar predictive power and adequate fit compared to the reference model. We used backwards elimination using the Wald test to remove predictors with P value < .10 starting with interaction terms and proceeding to the variable with the highest P value. We assessed the predictive power of reduced models due to removing variables or collapsing across categories by comparing the area under the receiver operating characteristic (ROC) curve. We evaluated model fit using the Hosmer-Lemeshow test (P value > .1) (129). We then created a scoring system from the logistic model output using the regression coefficient, to preserve the multiplicative nature of the score, for each predictor in the reduced model. We multiplied the regression coefficient by 10 and rounded to the nearest integer to simplify score use (118). A final score for each patient was obtained by summing the individual scores from their predictor values. To determine the utility of the scoring system, we evaluated the sensitivity, specificity, false negatives, false positives and the area under the ROC curve of different score cut-points. We calculated false negatives using the formula (1 − sensitivity) * gametocytemia prevalence * N and false positives as (1 − specificity) * (1 − gametocytemia prevalence) * N. We also calculated the percent of the population correctly classified and the percent of the population that would be treated if scores were used to target gametocytocidal therapy. We imported the final dataset into STATA (v10) and used it for all analyses.

Study power

Assuming a gametocytemia prevalence of at least 10% and $\alpha = .25$, we estimated more than 95% power in the study to detect risk factors prevalent among at least 7.5% of controls with a prevalence odds ratio of 2 or more.

Ethical clearance

The Scientific Advisory Committee of the National Institute of Malaria Research approved the original trials and the Institutional Review Board of the University of North Carolina approved the secondary analysis study.

RESULTS

During 2009 and 2010, 1,372 patients with *P. falciparum* malaria were recruited into therapeutic efficacy trials of antimalarial drugs. Among these patients, 19 voluntarily withdrew, 3 received outside treatment, 2 contracted other illnesses, and 9 were lost to follow-up. After removing 4 patients who were missing gametocytemia data our complete case population was 1,335. The majority of the study population was, independently, adult, male, from central India, and enrolled in the post-monsoon (Table 1). The proportion of patients with gametocytemia on day 0, day 1, and day 2 was 13% (n=179), 15% (n=201), and 15% (n=203) respectively. Overall, the prevalence of gametocytemia, i.e. gametocytes detected in blood films on any day from day 0 through day 2, was 19% (n=248) and this varied in relation to demographic and clinical classifications (Table 1). In the unadjusted bivariate associations, gametocytemia decreased with both increasing age and parasite density categories, while it increased among those without fever at enrollment or a history of fever prior to enrollment. Men and patients who reported yes or unknown previous antimalarial intake also had a higher prevalence of gametocytemia. The proportion of malaria

patients with gametocytemia varied by region and decreased along a western to eastern India axis.

The unadjusted prevalence of gametocytemia decreased from 26% (n=103) among ages 1-4 years to 14% (n=96) in those 50 years old or greater (Figure 1). Inversely, the proportion of the total parasite population consisting of gametocytes increased with age from 3% in 1-4 year olds to 8% in ages 50 or more years respectively. The average density, represented by the geometric mean, of the maximum gametocytemia and mean gametocytemia during days 0 through day 2 were 117 and 66 gametocytes/μL respectively. The density of gametocytes was higher in children compared to adults (Table 2) which was similar to the trend observed with enrollment asexual parasite density (data not shown). In unadjusted analysis, gametocyte densities were similar in western and central India but higher in northeast India in all age categories (Table 2). Adults (age 15 years or more), who were 54% of the study population and among whom 16% carried gametocytes, constituted approximately 44% of the reservoir for potential transmission. School-age children (age 5-15 years), who were 38% of the study population and among whom 20% carried gametocytes, constituted approximately 44% of the reservoir for potential transmission. Young children (age less than 5 years), who were 8% of the study population and among who 27% carried gametocytes, constituted approximately 12% of the reservoir for potential transmission. These estimates did not differ by region except for northeast India where the relative contributions of school-age children and younger children were reversed compared to other regions. These estimates also did not differ whether the maximum or mean gametocyte density was used.

Age, sex, the age-sex product interaction, region, previous antimalarial intake, fever at enrollment, and parasite density category remained in the reference model (Table 2). In the

simplified model age, sex, region, and previous antimalarial intake alone provided similar predictive ability and model fit (P value = .32) (Table S1). Possible risk scores ranged from 0 to 65 although the minimum and maximum observed score were 0 and 45 respectively. The median risk score was 14 (interquartile range: 10, 28). Residing in the western region was the highest scoring predictor with 28 points while age 5-14 years and male sex were the lowest scoring predictors receiving 4 points each. No cut-point yielded a sensitivity greater than 75% and a specificity lower than 75%. For example, if the goal of a control programme was to treat at least 90% of gametocyte carriers, a risk score of 14 or more provided 91% (95%CI: 88, 95) sensitivity and 33% (95%CI: 31, 36) specificity (Table 4). Applied in our study population of 1,335 patients of whom 248 were gametocytemic, 71% of the population would receive treatment with 22 false negatives and 723 false positives. The area under the ROC curve for predicting gametocytemia was 0.76 (95%CI: 0.73, 0.80) (Figure 2). For comparison, the AUC of the model using all predictors was 0.79 with 2-way interactions and 0.82 with all possible interactions.

DISCUSSION

We observed a high prevalence of gametocytemia in India and adults constituted a substantial proportion of the reservoir for potential transmission in our sampled population. While a predictive model for gametocytemia identified several easily screened risk factors, the ability of the clinical algorithm to sensitively and specifically detect gametocytemia was low.

We observed a higher prevalence of gametocytemia than previously reported although there were large, albeit unadjusted, regional differences. Gametocytemia was most common in western India which is composed of two distinct ecotypes: rural, low-

transmission malaria, and urban slum and migrant-labour associated malaria. Both ecotypes are conducive to the promotion of gametocytemia either through low immunity or poor access or quality of care received by migrants. Central India and northeast India are higher transmission areas with forest-associated malaria albeit through different vectors. Access to care in the northeast is higher and the use of artemisinin combination therapies began earlier which may explain the lower prevalence of gametocytemia in the patient population relative to central India (115). In Thailand the malaria control programme records the prevalence of gametocytemia, which arises 3-4 days after patency, as an indicator for monitoring access to care. Patients treated early in their disease course are less likely to carry gametocytes. Given the wide network of clinics with microscopy, India could also make use of gametocytemia in a similar fashion.

The reservoir for potential transmission in our study population was distributed throughout the age spectrum. Traditionally, children were thought to be the primary reservoir for transmission (25). Young and school-age children did contribute to the reservoir for potential transmission disproportionate to their population because of their higher prevalence of gametocytemia and higher gametocyte densities. Still, adults constituted nearly half of the potential reservoir of infection simply due to their larger population. In adults a higher proportion of the parasite population was gametocytes although this was largely due to a small denominator as the asexual parasite density decreased with age (data not shown). These results underscore the need to examine absolute measures of frequency rather than relative measures to inform public health conclusions. The contribution of adults in malaria transmission may be higher than the potential reservoir estimated by us when accounting for other factors such as their larger surface area for biting (130).

Four variables (region, age, sex, and previous antimalarial drug intake) predicted gametocytemia in our model. Previous intake of non-gametocytocidal antimalarial drugs is thought to induce 'stress' on the parasite which activates gametocytogenesis (131). Age, sex, and region would, presumably, be associated with gametocytemia through one of two mechanisms: 1) immunity, primarily determined by transmission intensity and the exposure of specific risk-groups and 2) treatment access or treatment seeking behavior as gametocytemia increases with longer durations of infection (132). Parasite density and fever at the time of enrollment, which were removed from the final model, are also distal effects of, rather than proximal markers of, the aforementioned mechanisms which may explain their inability to predict gametocytemia in our model.

The use of a predictive model to detect gametocytemia generated an algorithm which ranked a positive case selected at random from our study population higher than a negative case selected at random 76% of the time (AUC). No cut-point yielded an acceptable sensitivity (>95%) and specificity (>90%) according to criteria developed for malaria rapid diagnostic tests (133,134). As an illustration, if we selected 90% sensitivity or more as a desirable criterion, we could only achieve 33% specificity. We also did not validate our algorithm on independent data and hence, its performance in our study population could be considered a best case scenario. Alternative strategies for selecting a predictive model are unlikely to produce better clinical algorithms with the available data as the AUC of the final model was close to the AUC of the saturated model. Alternative data however, could produce better clinical algorithms if other easily measured predictors existed. While our performance would not suffice for a disease diagnostic, one could argue the direct costs of using an algorithmic approach are non-existent so any reduction of false-positives is a benefit compared to universal treatment. However, substantial indirect costs may exist.

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Considerations of implementing any clinical algorithm must account for the operational challenges in individual level targeting including the costs of training, the time required for patient assessment, and increased programme complexity. The poor prospects for future improvement in model performance coupled with the likelihood of considerable indirect costs of implementation suggests a clinical predictive approach for targeting gametocytemia is not viable.

Our study had several limitations. We used microscopy for the measurement of gametocytemia which is less sensitive than molecular techniques. However, in studies comparing the two methods, the latter increased the magnitude of gametocytemia but did not alter its age-structure, circulation time, and other trends (13,28). Interpreting the functional relevance of submicroscopic gametocytemia is also difficult. While sub-microscopic density infections can infect mosquitoes, the probability of infection, the proportion of mosquitoes infected, and the density of infection in mosquitoes is positively correlated with gametocyte density (48,52). Next, we completed enrollment at each site over 1 to 2 months which restricted the analysis of seasonal trends of gametocytemia. In any given area, most patients were enrolled when malaria transmission was the most intense and vector control interventions in India are normally timed to dampen any seasonal oscillations. Our population cross-section was also not representative of the population at-risk. However, it is representative of the population encountered by the control programme through active and passive case detection. Thus, we did not assess the contribution of asymptomatic carriers to transmission but, at present, there may be no valid means to do so (122). The population was appropriate for our goal - to observe the population amenable to current and future interventions. Finally, we used the presence of gametocytes in peripheral blood as a proxy for infectiousness. Infectivity is modified by a number of factors and its direct assessment

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through membrane-feeding experiments, that are labour and time intensive, would have precluded the type of large survey needed for generalizable results.

In a population of *P. falciparum* patients from a national network of sentinel sites, we conclude gametocytemia was common, adults were an important component of the reservoir for potential transmission, and clinical algorithms based on predictive modeling were not effective for the detection of gametocytemia. Due to the wide age-distribution of gametocytemia, and the difficulty of targeting using clinical prediction, we recommend universal application, if any, of gametocytocidal interventions among confirmed malaria patients. Future research on gametocytemia could try to measure the asymptomatic reservoir, conduct longitudinal assessments, and validate gametocytemia as an indicator for treatment access.

TABLE 5.1. Prevalence and association of gametocytemia in relation to demographic and clinical factors of patients from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

Characteristic	Gametocytemia		Total	
	n	Row %	n	Col %
Age (years)				
<5	28	27	105	8
5-14	101	20	503	38
≥15	119	16	727	54
Sex				
Male	157	20	773	58
Female	91	16	562	42
PAI				
No	241	18	1,310	98
Yes/unknown	7	28	25	2
Region				
Central	84	11	731	55
Western	153	41	371	28
Northeast	11	5	233	17
Fever day 0				
Yes	144	16	929	70
No	104	26	406	30
Season				
Monsoon	34	8	406	30
Post-monsoon	174	28	620	46
Winter	40	13	309	23
PD (#/μL)				
<5,000	118	25	480	36
5,000-49,999	117	16	746	56
≥50,000	13	12	109	8
History of fever				
Yes	237	18	1,317	99
No	11	61	18	1

Abbreviations: PAI, previous antimalarial intake; PD, asexual parasite density; POR, prevalence odds ratio; CI, confidence interval; Col, column;

TABLE 5.2. The contribution of age groups to the reservoir for potential transmission using the maximum or mean day zero to three gametocyte density in patients from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

Region	Age (years)	N	%G	Maximum		Mean	
				GD	PR	GD	PR
Central	<5	64	20	87	0.11	46	0.11
	5-14	301	11	141	0.43	77	0.45
	≥15	366	10	130	0.46	65	0.44
Western	<5	13	77	152	0.09	24	0.04
	5-14	136	49	122	0.49	47	0.50
	≥15	222	35	88	0.42	37	0.46
Northeast	<5	28	18	208	0.36	153	0.46
	5-14	66	3	120	0.08	84	0.10
	≥15	139	3	402	0.56	182	0.44
Total	<5	105	27	124	0.12	69	0.12
	5-14	503	20	128	0.45	71	0.45
	≥15	727	16	102	0.43	58	0.43

Abbreviations: %G, prevalence of gametocytemia; GD, geometric mean gametocyte density per microliter; PR, proportion of the reservoir for potential transmission

TABLE 5.3. Adjusted prevalence odds ratios in the reference and final models, regression coefficients, and risk scores for predicting gametocytemia in patients from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

Variable	Reference model OR (95%CI) AUC=0.766		Final model OR (95%CI) AUC=0.762		Logistic regression coefficient	Risk score
Age (years), Sex						
<5, male	2.34	1.03, 5.28	3.88[a]	2.13, 7.06	1.36	14
<5, female	6.88	3.05, 15.6				
5-14, male	1.30	0.79, 2.14	1.51[a]	0.96, 2.36	0.41	4
5-14, female	2.07	1.18, 3.64				
≥15,male	2.07	1.26, 3.40	1.00[a]			
≥15, female	1.00					
Sex						
Male	--		1.49	1.06, 2.10	0.40	4
Female			1.00			
PAI						
No	1.00					
Yes/unknown	1.69	1.00, 2.87	1.67	0.99, 2.81	0.51	5
Region						
Central	2.98	1.69, 5.28	2.77	1.63, 4.69	1.02	10
Western	16.3	9.44, 28.1	17.1	9.98, 29.3	2.84	28
Northeast	1.00					
Fever day 0						
Yes	1.30	0.93, 1.81	--			
No	1.00					
PD (#/μL)						
<5,000	1.54	0.74, 3.24	--			
5,000-49,999	1.44	0.72, 2.88				
≥50,000	1.00					

[a]for the entire age category
Abbreviations: PAI, previous antimalarial intake; PD, asexual parasite density; OR, odds ratio; CI, confidence interval

TABLE 5.4. Performance of different risk score cut-offs for detecting gametocytemia in patients from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

Score ≥	Sensitivity	Specificity	Number of FN	Number of FP	Percent treated
0	1.00	0.00	0	1087	100
4	1.00	0.03	1	1059	98
8	0.98	0.15	5	924	87
10	0.98	0.18	6	888	85
13	0.92	0.32	20	738	72
14	0.91	0.33	22	723	71
15	0.76	0.59	59	441	47
17	0.76	0.60	60	435	47
18	0.75	0.61	63	427	46
19	0.67	0.75	81	277	33
23	0.67	0.75	82	270	33
27	0.63	0.78	91	242	30
28	0.62	0.80	95	219	28
32	0.57	0.85	107	165	23
33	0.19	0.97	200	32	6
36	0.19	0.97	200	31	6
37	0.06	1.00	233	5	1
41	0.05	1.00	235	3	1
45	0.02	1.00	244	2	0
46	0.00	1.00	248	0	0

Abbreviations: FN, false negative; FP, false positive

TABLE 5.S1. Cross-tabulation according to predictors identified in the reduced model of patients from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

Sex	Region	Northeast						Central						Western					
	Age	<5		5-15		>15		<5		5-15		>15		<5		5-15		>15	
	PAI	g	n	g	n	g	n	g	n	g	n	g	n	g	n	g	n	g	n
Male																			
	No	2	17	1	29	1	29	9	36	15	131	14	164	6	7	29	73	12	66
	Yes	0	0	0	0	0	0	0	0	0	1	1	7	0	0	1	1	0	1
Female																			
	No	3	11	1	37	3	110	4	27	18	168	22	188	4	6	33	59	64	152
	Yes	0	0	0	0	0	0	0	1	0	1	1	7	0	0	3	3	1	3

Abbreviations: G, number gametocytemic; PAI, previous antimalarial drug intake

FIGURE 5.1. Prevalence of gametocytemia and the percent of total parasites that were gametocytes by age category of patients from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010
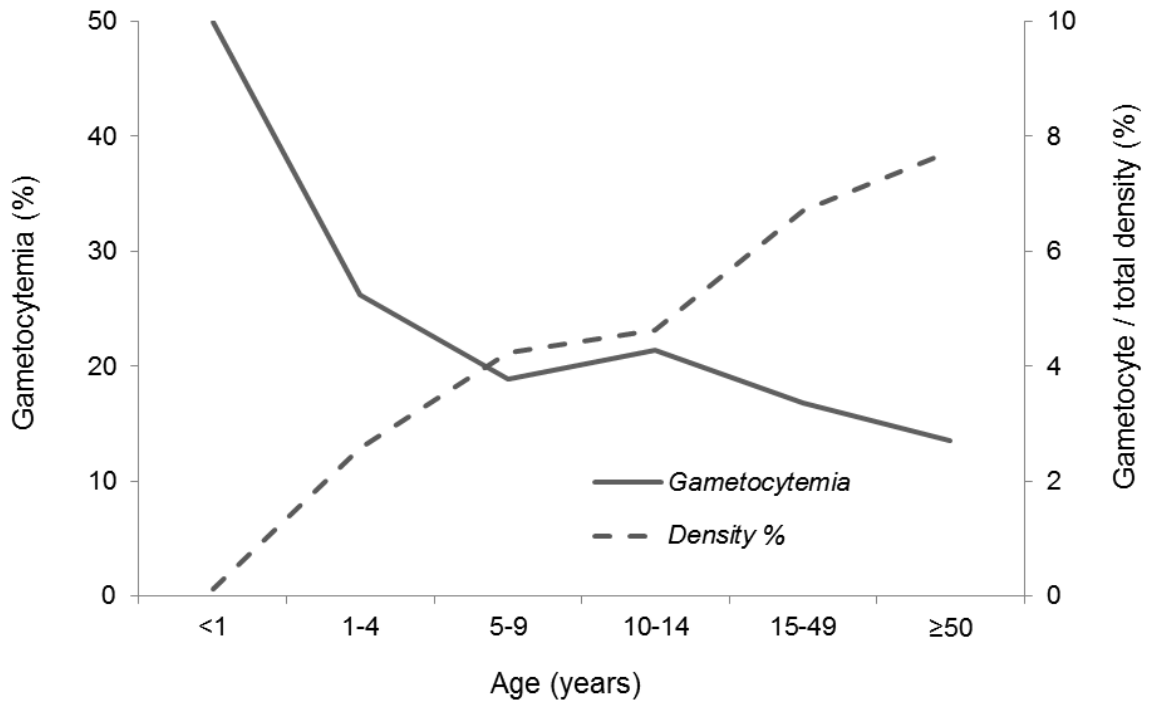
FIGURE 5.2. Receiver operator characteristics curve with risk score cut-points for predicting gametocytemia in patients from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010
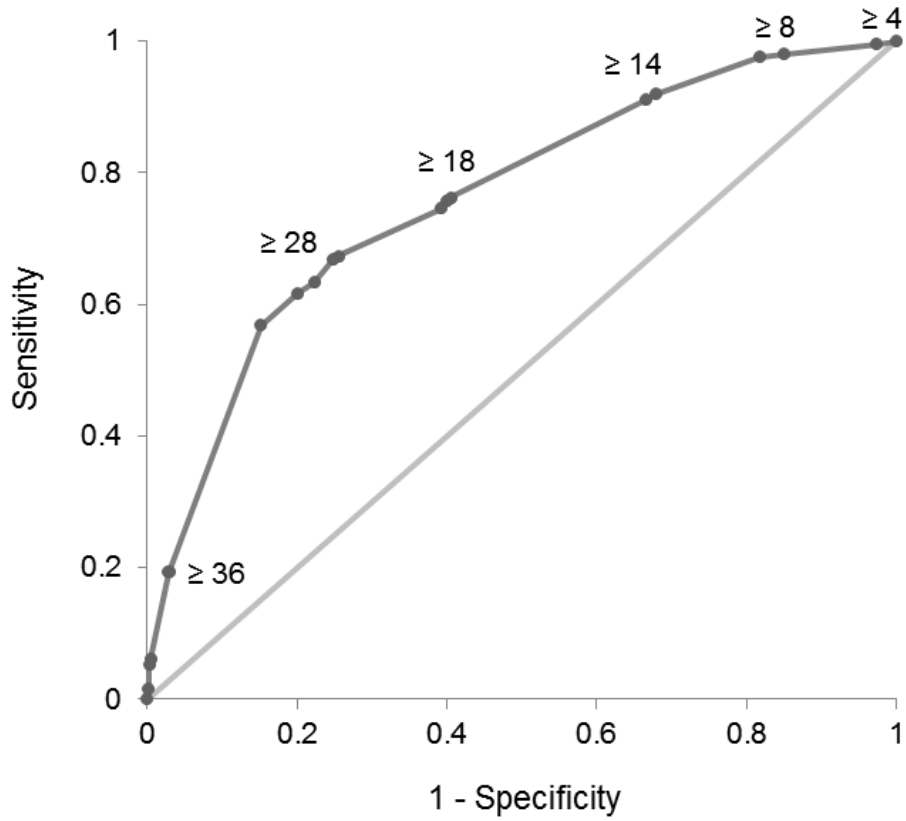
CHAPTER SIX: A NON-RANDOMIZED CONTROLLED TRIAL OF ARTESUNATE PLUS SULFADOXINE-PYRIMETHAMINE WITH OR WITHOUT PRIMAQUINE FOR PREVENTING THE POST-TREATMENT TRANSMISSION OF *PLASMODIUM FALCIPARUM*

INTRODUCTION

The primary goal of antimalarial drug therapy is to reduce morbidity and mortality by achieving clinical and parasitological cure. A crucial secondary aim however, is to reduce the infectious reservoir and decrease malaria transmission. Gametocytes are the sexual stage of *Plasmodia* life cycle that render malaria patients infectious to mosquitoes and propagate transmission. Treatment considerations for clearing gametocytes are distinct from those for asexual stages because of the stage-specific actions of antimalarial drugs, particularly for *P. falciparum*. Most antimalarial drugs target the asexual blood stages responsible for disease. The sensitivity of gametocytes to most antimalarial drugs is less than that of asexual stages. Among gametocytes, the sensitivity of mature stages is less than that of immature stages. Chloroquine, quinine, and sulfadoxine-pyrimethamine for example, are active against newly differentiated gametocytes but lose potency as gametocyte development progresses (53,55).

The chemotherapeutic control of gametocytes is largely achieved through antimalarial treatment with effective blood-stage schizonticides (135). Resolving the asexual parasitemia prevents the generation of new gametocytes and limits the period of infectivity to the circulation time of existing gametocytes. However, gametocytemia following treatment may be prevalent in a high proportion of patients and persist approximately for up to a month. Eliminating the post-treatment infectious period could provide substantial impact,

particularly in low to moderate endemic countries where the size of the infectious reservoir is a critical determinant of transmission. The marginal direct and indirect costs of adjunct gametocyte-specific treatment during the case management process make such a strategy attractive.

Artemisinin is active against all gametocyte stages barring those fully mature, and this characteristic contributed to the impressive declines in transmission following its widespread implementation in several countries (58). Still, in trials with artemisinin combination therapy (ACT) mature gametocytes persisted in 10-30% of patients and remained infectious to mosquitoes (51,61,62). Primaquine, which has no activity against asexual *P. falciparum* parasites, is effective against mature gametocytes (63,65). A single-dose of primaquine has been used as an as an adjunct treatment, for its gametocytocidal effect, alongside first-line therapies in many countries since the 1960s (66,67). The key question for *P. falciparum* treatment is whether a single dose of primaquine should be added to ACTs to further reduce the transmissibility of the treated infection (136). Recently, the Malaria Policy Advisory Committee of the World Health Organization (WHO) noted the gametocytocidal use of primaquine in the treatment for *P. falciparum* as a priority question for operational research (137).

The control of malaria is a key challenge for India which reported 1.5 million cases in 2010 from extensive routine surveillance (126). The reduction of transmission is a priority with most of the country in areas of low and moderate malaria endemicity. Before the switch to artesunate plus sulfadoxine-pyrimethamine (AS+SP) in 2005, the use of single-dose primaquine as a gametocytocide was part of the national drug policy for *P. falciparum* treatment in the country since the malaria eradication era (100). With the addition of an ACT to the drug schedule, primaquine's role was uncertain as there was little evidence of the

efficacy or safety of using both. WHO recommendations were, at the time, ambivalent (106) so national policy did not specify single dose primaquine use or disuse (107). In late 2010 primaquine was added to the first-line treatment based on new recommendations but evidence from trials in India is needed to support the policy.

The goal of this study was to estimate the gametocytocidal effect of adding primaquine to ACT in India. We conducted a secondary analysis of data from a national network of sentinel sites for monitoring antimalarial drug resistance. The study protocols did not contain instructions on primaquine use as there were no policy guidelines at the time of the study. The use of the drug by some sites and not in others created a natural experiment. First, we compared the clinical and demographic characteristics of patients among the groups to determine if the populations were similar. Second, we computed crude and adjusted measures of effect for post-treatment clearance (gametocyte survival) and post-treatment carriage (gametocyte circulation) to estimate the additional effect of primaquine. Third, we conducted a sensitivity analysis of the measurement of patient follow-up time to assess whether our estimates were robust to its misclassification. Finally, we compared reported adverse events among the groups to assess safety and tolerability.

METHODS

Study sites and population

We utilized data from 22 *P. falciparum* therapeutic efficacy trials conducted through the National Antimalarial Drug Resistance Monitoring Network of India in 2009 and 2010 (114). Among the sites, 9 used primaquine along with AS+SP while 13 used AS+SP alone. We could not obtain data from 1 site (Gadcharoli, no primaquine use) because gametocytemia was not recorded in case record forms and slides could not be re-examined.

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The National Vector Borne Disease Control Programme and the National Institute of Malaria Research purposively selected the study sites to represent *P. falciparum* transmission settings across the country. We included all patients eligible for the WHO therapeutic efficacy trial protocol: patients with *P. falciparum* monoinfection, febrile or with a history of fever, asexual parasite density greater than 500/µL and less than 100,000/µL, and willingness to consent to follow-up. We excluded pregnant patients and those with signs of severe malaria, and patients less than one year of age as primaquine is contraindicated in this group. The purpose of these trials was to measure the clinical and parasitological response of malaria patients when treated with first-line drugs used in national policy. The study population represents a cross-section of the patients who presented to the local clinic or were recruited through active case detection in nearby communities.

Data collection

The data collection methods have been previously described (114). Briefly, clinical and demographic information were recorded from each patient at enrollment. Patients were followed during the first 3 days of treatment and then at weekly intervals from enrollment until day 28. Patients received AS+SP (4mg/kg for three days plus 25/1.25mg/kg) as per national guidelines with or without primaquine on the third day of treatment (0.75mg/kg, usual adult dose of 45mg). At each follow-up visit a physical exam was conducted and thick and thin blood smears were prepared. Using routine oil immersion reading at 1,000X on Giemsa-stained thick smears, slides were counted until 200 white blood cells (WBC) if gametocytes and asexual stages were present or until 1,000 WBC to declare a slide negative for both. A count of 8,000 WBC per µL of blood was assumed to obtain a final parasite density. The data were double-entered into the WHO therapeutic efficacy database and blood slides were cross-checked by expert microscopists. Existing therapeutic efficacy data was

consolidated into a single database after range checks to investigate plausible values for each data field.

Case and predictor definitions

Primaquine therapy was the main exposure. We obtained primaquine use information from the site physician. While site specific practices differed, physicians indicated they administered primaquine uniformly, i.e. to all patients or none, within sites so group exposure was used. We defined pre-treatment gametocytemia as the presence of gametocytes in peripheral blood on any day between day 0 and day 2 in a patient eligible for a therapeutic efficacy study in 2009 or 2010. We defined post-treatment gametocyte clearance as the first day without gametocytemia after day 2 in a patient with pre-treatment gametocytemia. We defined an event of post-treatment gametocytemia as the presence of gametocytes on any follow-up day after day 2 in a patient eligible for the therapeutic efficacy study in 2009 or 2010. We excluded gametocytemia at the time of failure for patients with recrudescence or re-infection. For measuring exposure time in weeks we censored at the end of the interval. We reported the data in person-weeks, instead of person-days, to enable comparison with previous literature. For the measurement of post-treatment gametocyte circulation, patients who completed follow-up accrued an additional week of exposure in order to incorporate the contribution of individuals who remained positive at the end of day 28. Thus, the total post-treatment exposure time of 32 days is congruent with biological estimates of the maximum circulation time of gametocytes (13).

We compared covariates associated with gametocytemia in prior literature: age, sex, season, region, previous antimalarial drug intake, current fever, history of fever, and asexual parasite density (15). We categorized age using cutoffs corresponding to age-wise treatment blister packs (<1, 1-4, 5-9, 10-14, and ≥15 years categories) and asexual parasite density

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using cut-offs used in previous studies (<5,000, 5,000-9,999, 10,000-49,999, and ≥50,000 parasites/μL). The recall period for history of fever was the past 48 hours and for taking an antimalarial drug was the past week. We defined current fever as an axillary temperature ≥37.5°C at the time of enrollment. We designated region using geographic clusters associated with different malaria ecotypes: western India as Gujarat, Mumbai, and Rajasthan, central India as Andhra Pradesh, Chhattisgarh, Gadcharoli, Jharkhand, Madhya Pradesh, and Orissa, and northeast India as the Assam, Meghalaya, and West Bengal sites (85,115). Multiple ecotypes, each with different implications for both transmission intensity and malaria control strategy, exist within each of these broad regions (127). However, the categorization captures the dominant ecotype, e.g. forest malaria in central India, or agglomerates overlapping ecotypes of not so dissimilar transmission intensity as with rural, agricultural malaria and urban, migrant malaria in western India. Finer resolution was not possible without a loss of power. We classified season by month of enrollment: monsoon – June-August, post-monsoon – September-November, and winter – December and January.

Data analysis

We analyzed all patients who completed treatment. None of the covariates, age, sex, season, region, previous antimalarial drug intake, current fever, history of fever, and asexual parasite density, had missing data. Missing gametocytemia data, due to withdrawal and loss to follow-up during the treatment phase, was less than 3% of the overall sample. We tabulated the clinical and demographic characteristics, as well as the primary and secondary study outcomes, of patients who received and did not receive primaquine. We collapsed categories of continuous variables where the prevalence of gametocytemia was similar among adjoining groups. We calculated the mean and median gametocyte clearance times by treatment group and compared them using the t-test and ranksum tests. We calculated the

incidence, incidence rate ratios, and incidence rate differences over follow-up time among the primaquine and no primaquine groups.

We modeled the effect of primaquine by estimating the post-treatment clearance rates of gametocytes among patients with gametocytemia at enrollment and by comparing the circulation of post-treatment gametocyte-weeks among the entire population. We estimated crude and adjusted relative measures of effect, hazard ratios and incidence rate ratios respectively, as these were readily interpretable and generalizable across our diverse study sites. We used cox regression with the Efron method for ties (119) with clustered (on site) robust standard error and Poisson regression using general estimating equations with unstructured correlation matrixes and robust clustered (on patient) standard error respectively. We constructed a causal diagram of the effect of primaquine on gametocytemia to identify minimally sufficient and sufficient adjustment sets using the DAG program v2.1 (http://epi.dife.de/dag). Based on our diagram, adjustment on district was sufficient to control confounding bias. However, we controlled for region instead since the exposure, primaquine, did not vary by district. To assess the robustness of the measure of effect to uncontrolled confounding, we also produced estimates adjusted for age and parasite density, variables strongly associated with gametocytemia, as well as adjusted for all covariates whose distribution differed between the two arms (*P* value < .10). We included an interaction term between region and primaquine and assessed effect measure modification if the Wald test for the interaction term was *P* value < .2. We used a backwards elimination strategy to retain confounders that changed the estimate by at least 10%. In the cox model we compared the model with and without discrete time (marginal) due to the large number of tied events and assessed the proportional hazards assumption by examining the -log(log(survival)) over time for parallel trends between the groups. We compared the Poisson model to a negative

binomial model using a likelihood ratio test with criteria *P* value < .05 to ensure appropriate

fit with outcome dispersion.

The time at risk was measured in intervals and not continuously, i.e. on day 7, day 14,

etc. A patient gametocytemic at day 7 and not gametocytemic on day 14 may have cleared

gametocytes at any point during the interval which potentially misclassifies the exposure

time. We used the same time within the interval to censor for both groups which assumed no

difference in inter-interval clearance and could underestimate the measure of effect. The

inter-interval time likely differs by primaquine status since the drug accelerates gametocyte

clearance. Based on previous studies, we conducted a sensitivity analysis assuming 50%

reduction in time at risk in a given interval among primaquine exposed patients. We plotted

the mean area under the curve (AUC) for gametocyte density for each group by study visit.

We excluded outliers of high pre-treatment gametocyte density (maximum > 2,000/μL, n=5)

that disproportionally influenced the AUC. We calculated the AUC per day using the formula

of Mendez et al. with the addition of the area for gametocytemia remaining at the end of

follow-up (120). We compared the median AUC per day for each group using the ranksum

test. We imported the final dataset into STATA (v10) and used it for all analyses.

Study power

For gametocyte clearance, our sample size of approximately 230 patients with pre-

treatment gametocytemia and $\alpha = .05$ provides more than 90% power to detect a hazard ratio

of 0.5 assuming 10% withdrawal and 80% of patients experiencing the outcome during the

follow-up period. With a no primaquine to primaquine ratio of approximately 2:1, $\alpha = .05$,

and assuming the prevalence of gametocytemia among the exposed as 5%, we calculated

more than 90% power to detect a gametocyte-week incidence rate ratio greater than 2.

Ethical clearance

The Scientific Advisory Committee of the National Institute of Malaria Research approved the original trials and the Institutional Review Board of the University of North Carolina approved the secondary analysis.

RESULTS

During 2009-2010, 1,372 eligible patients were recruited among 21 sites (Figure 1). Nine sites where 543 patients completed treatment used primaquine while eleven sites where 796 patients completed treatment did not use primaquine. In each group 2 patients missing gametocytemia data were excluded; the final complete case population was 1,335 patients (541 primaquine, 794 no primaquine). Among treatment failures, 4 of 12 recrudescent patients, 0 of 2 *P. falciparum* re-infection patients, and 0 of 6 *P. vivax* re-infection patients were gametocytemic at the time of failure. The clinical, demographic, and treatment efficacy as well as parasite clearance outcomes were similar between study groups with the exceptions of a higher proportion of males, adults, and patients from northeast India in the primaquine unexposed group (Table 1). Critically, the pre-treatment gametocytemia prevalence in the no primaquine group (18%, n=141) was similar to the primaquine group (20%, n=107). The prevalence of gametocytemia between the arms was similar on days 0, day 1, and day 2 and subsequently declined among the primaquine exposed arm starting on day 3 (Figure 2).

Among 248 patients with pre-treatment gametocytemia, the median time to gametocyte clearance was 7 days (interquartile range: 3, 14) in patients who received primaquine and 14 days (interquartile range: 3, 28) in patients who did not (*P* value < .001). Primaquine with AS+SP cleared gametocytes faster than AS+SP alone with only 3% survival

of gametocytemia compared to 23% at the end of follow-up (Figure 3). Using cox regression the crude hazard ratio for the effect of primaquine on the rate of gametocyte clearance was 2.2 (95%CI: 1.2, 4.2) over 28 days. Adjusted for region, the addition of primaquine resulted in an increased rate of gametocyte clearance 1.9 times (95%CI: 1.1, 3.3) that of AS+SP alone over 28 days. The effect measure for primaquine was robust to adjusting for age and parasite density or all covariates selected by univariate associations (Table S1). We did not detect any modification of the effect with time or region.

The development of post-treatment gametocytemia among patients who were pre-treatment negative for gametocytemia was lower (0.7%, 3/434) in the primaquine group compared to the no primaquine group (2.8%, 18/653) (*P* value = .02). Primaquine reduced the absolute and relative rates of post-treatment gametocyte circulation among the population (Table 2). Using Poisson regression with general estimating equations, the incidence rate ratio for the effect of primaquine on the number of post-treatment gametocyte-weeks was 0.3 (95%CI: 0.2, 0.6). Adjusted for region, primaquine decreased the number of post-treatment gametocyte weeks by 45% (95%CI: 19, 62). The effect measure for primaquine was robust to adjusting for age and parasite density or all covariates selected by univariate associations (Table S1). We did not detect any modification of the effect with region or over-dispersion of the outcome.

In our sensitivity analysis assuming faster intra-interval clearance/exposure time, the estimated effect of primaquine increased compared to the previous measures produced with more conservative assumptions (Table S2). The crude and region adjusted hazard ratio for primaquine on the rate of gametocyte clearance was 2.8 (95%CI: 1.5, 5.5) and 2.5 (95%CI: 1.4, 4.3) over 28 days. The crude and region adjusted incidence rate ratio for primaquine on the number of gametocyte-weeks was 0.2 (95%CI: 0.1, 0.3) and 0.3 (95%CI: 0.2, 0.4) over

28 days. The mean AUC of each group at a study visit decreased over time compared to no primaquine (Figure 4). The median AUC per day for both groups was 0 gametocytes/µL due to the high proportion of patients without post-treatment gametocytemia. The 90th-99th percentile AUC per day was 2.2-79 gametocytes/µL for primaquine and 9.1-198 gametocytes/µL for AS+SP alone (*P* value = .01).

Adverse events reported during follow-up included vomiting (8) and fever (2) in the AS+SP alone arm and vomiting (14) and jaundice (1) in the primaquine arm. Only 5 of the 14 patients who experienced vomiting did so on the day of primaquine administration. The case of jaundice occurred in a 5-year-old, male child on day 14.

DISCUSSION

The addition of primaquine to AS+SP reduced the potential of post-treatment transmission of *P. falciparum* in India. The gametocytocidal dose of primaquine increased gametocyte clearance by one week in patients who were positive pre-treatment, decreased the incidence of gametocytemia in patients who were negative pre-treatment, and was well tolerated.

We observed a high potential for *P. falciparum* transmission after treatment with an ACT alone. The prevalence of gametocytemia at the end of follow-up for those receiving only AS+SP was 5% among all patients and 24% among those initially positive for gametocytes. While the choice of partner drug may also affect post-treatment gametocytemia prevalence, as SP monotherapy is particularly gametocytogenic (38), our observations were similar to those observed for other combination therapies elsewhere (138). We measured the effect of primaquine through gametocyte clearance, the number of gametocyte-weeks of circulation, and the area under the curve using gametocyte density over time. By any

approach, the addition of primaquine provided substantial benefit even in the presence of an artemisinin based treatment. Our sensitivity analysis suggests our estimates were conservative and the effect of primaquine may be larger. Other estimates of the effect of primaquine with an ACT include the 92% reduction in gametocyte-weeks in Burma (75), 83% reduction in the prevalence on any day of follow-up in Tanzania (47), and the acceleration of gametocyte clearance by a week in Colombia (76). While our estimate of clearance time was similar to the estimate from the low transmission setting of the Colombian trial, the reduction of post-treatment gametocytemia was lower than the other two studies in high transmission areas. The difference in primaquine effectiveness could be due to the different host immunity or pharmacokinetics of the study population, but there is also the possibility of reduced primaquine efficacy in India. Since the extensive malaria eradication program in the 1960s, India has a long history of primaquine use. Several studies previously reported the frequent persistence of gametocytemia following primaquine administration (97,113) and slower clearance of gametocytes after treatment with primaquine compared to a novel 8-aminoquionline in Western India (98).

   While primaquine certainly works, several operational questions remain regarding its safety, optimal dose, and the day of administration. The primary concern with primaquine use is safety given the risk of haemolysis in individuals with G6PD deficiency (63). Notably, most severe events have been associated with the longer primaquine dosing used for anti-relapse treatment in *P. vivax* and not the gametocytocidal single-dose. In previous single-dose primaquine trials, haemoglobin concentrations among exposed patients were 5% lower at day 7 (recovered by the end of the month) or the mean increase over the study was 0.3g/dL less compared to patients who only received ACT (47,75). Recent research also suggests haemolysis may occur in non-G6PD individuals as well though severe anemia was rare (71).

The only other adverse effect attributable to primaquine in any study was increased

abdominal pain (75,76). However, haemolysis and side-effects will vary by population and in

the context of already vulnerable populations (74) more assessments, particularly post-

marketing pharmacovigilance, are needed. The current dose of primaquine was determined

through limited experiments in the 1960s (66,67). Lower, or higher, doses of primaquine may

be equally, or more, effective as well as safe – Thailand, for example, uses a 30mg adult dose

of primaquine for gametocytocidal therapy (60). A 4-arm, randomized study of different

doses is underway in Uganda and should help determine the trade-offs, if any, between

primaquine efficacy and safety (139). The day of primaquine administration varies from day

0 to day 2 with little evidence available to guide policy. For day 0, treatment with primaquine

can be observed but in non-fixed-dose therapies, such as AS+SP, patients already suffer a

large pill burden. For day 3, the prospect of eliminating mature gametocytes is the highest

since the window of exposure is short and treatment would be occurring, most likely, after

the completion of asexual parasite clearance. Modeling studies have suggested later

administration a week after ACT treatment may have the most transmission reducing effect

(140). However, such a regimen could be operationally difficult to implement.

Our study had several limitations. We used microscopy for the measurement of

gametocytemia which is less sensitive than molecular techniques. However, in studies

comparing the two methods, the latter increased the magnitude of gametocytemia but did not

alter its age-structure, circulation time estimates, or other trends (13,28). Interpreting the

functional relevance of submicroscopic gametocytemia is also difficult. While sub-

microscopic density infections can infect mosquitoes, the probability of infection, the

proportion of mosquitoes infected, and the density of infection in mosquitoes is positively

correlated with gametocyte density (48,52). Second, primaquine use was not randomized.

While we controlled for several sets of covariates and assessed effect measure modification, confounding due to unmeasured causes may bias our estimates and the possibility of heterogeneity of the effect cannot be excluded with the available power (123). Third, initial gametocytemia among patients may have varied depending on the form of their recruitment. The direction of bias is unclear as active case detection could detect patients earlier in their disease course prior to self-referral or later in the disease course post symptom attenuation, compared to passive case detection. Finally, we used the presence of gametocytes as a proxy for infectiousness. Infectivity is modified by a number of factors and its direct assessment through membrane-feeding experiments, that are labour and time intensive, would preclude large, multi-site trials needed for generalizable results.

We conclude primaquine reduced the potential for post-treatment transmission of *P. falciparum* compared to AS+SP alone. Single-dose primaquine could be provided along with ACT in India to improve malaria control. We outline several avenues of future operational research to needed for optimizing the adjunctive use of primaquine.

TABLE 6.1. Demographics, clinical characteristics, and primary outcomes by primaquine receipt status of patients from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

Characteristic	Value	No primaquine n=794	%	Primaquine n=541	%
Region	Central	312	39	419	77
	Western	249	31	122	23
	Northeastern	233	29	0	0
Sex	Male	506	64	267	49
	Female	288	36	274	51
Age category (years)	0-4	44	6	61	11
	5-9	112	14	153	28
	10-14	131	16	107	20
	15-49	445	56	186	34
	≥50	62	8	34	6
Asexual parasite density / µL	<5,000	290	37	190	35
	5,000-10,000	144	18	86	16
	10,000-50,000	308	39	208	38
	≥50,000	52	7	57	11
Febrile (≥37.5°C)	No	249	31	157	29
	Yes	545	69	384	71
History of fever	No	18	2	0	0
	Yes	776	98	541	100
Previous drug intake	No	781	98	529	98
	Yes	8	1	9	2
	Unknown	5	1	3	1
Primary classification	ACPR	765	96	527	97
	ETF	2	0	1	0
	LCF	5	1	1	0
	LPF	9	1	3	1
	LFU	13	2	7	1
	WTH	0	0	2	0
Parasite clearance	≤24 hrs	468	59	338	62
	24 - 48 hrs	215	27	137	25
	48 - 72 hrs	93	12	61	11
	≥72 hrs	16	2	5	1
Pre-treatment gametocytemia	No	653	82	434	80
	Yes	141	18	107	20

Abbreviations: PQ, primaquine; IR, incidence rate; IRR, incidence rate ratio; IRD, incidence rate difference

TABLE 6.2. Post-treatment gametocyte circulation by primaquine group among patients from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

Unit	No PQ		PQ		IRR	95%CI	IRD	95%CI
	N	IR	N	IR				
Gametocyte-weeks	285	7.97	92	3.78	0.47	0.37, 0.60	-0.042	-0.030, -0.054
Person-weeks	3,574		2,436					

Abbreviations: PQ, primaquine; IR, incidence rate per 100-person-weeks; IRR, incidence rate ratio; IRD, incidence rate difference; CI, confidence interval

TABLE 6.S1. Crude and adjusted measures of effect for primaquine on post-treatment gametocyte clearance rates and post-treatment gametocyte circulation using different modeling and coding options among patients from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

Model	Covariates	Efron ties		Discrete ties (m)	
		HR	95%CI	HR	95%CI
Cox		2.20	1.15, 4.23	2.27	1.72, 3.01
Cox	Region	1.89	1.07, 3.32	1.93	1.44, 2.60
Cox	Age, PD	2.05	1.27, 3.33	2.13	1.56, 2.90
Cox	All	1.69	1.02, 2.81	1.74	1.27, 2.38
Model	Covariates	Person-days (nb)		Person-weeks (p)	
		IRR	95%CI	IRR	95%CI
GEE		0.38	0.25, 0.59	0.34	0.20, 0.58
GEE	Region	0.56	0.37, 0.83	0.55	0.38, 0.81
GEE	Age, PD	0.44	0.29, 0.66	0.32	0.17, 0.60
GEE	All	0.72	0.47, 1.12	0.69	0.46, 1.01

Abbreviations: Region, reduced region with Northeast/Central collapsed; Age, age in years; PD, log parasite density; All, age-PD-sex-history of fever- Region; NB, negative binomial; P, Poisson; M, marginal; CI, confidence interval

TABLE 6.S2. Sensitivity analysis of crude and adjusted measure of effect for primaquine on post-treatment gametocyte clearance rates and post-treatment gametocyte circulation assuming 50% faster interval-clearance for primaquine recipients among patients from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

Covariates	Clearance		Circulation	
	HR	95%CI	IRR	95%CI
	2.82	1.46, 5.46	0.15	0.08, 0.29
Region	2.47	1.41, 4.32	0.27	0.18, 0.39
Age, PD	2.69	1.64, 4.40	0.17	0.09, 0.31
All	2.22	1.37, 3.75	0.35	0.23, 0.52

Abbreviations: Region, reduced region with Northeast/Central collapsed; Age, age in years; PD, log parasite density; All, age-PD-sex-history of fever- Region; CI, confidence interval

FIGURE 6.1. Flow-chart of patients eligible for treatment with primaquine from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010
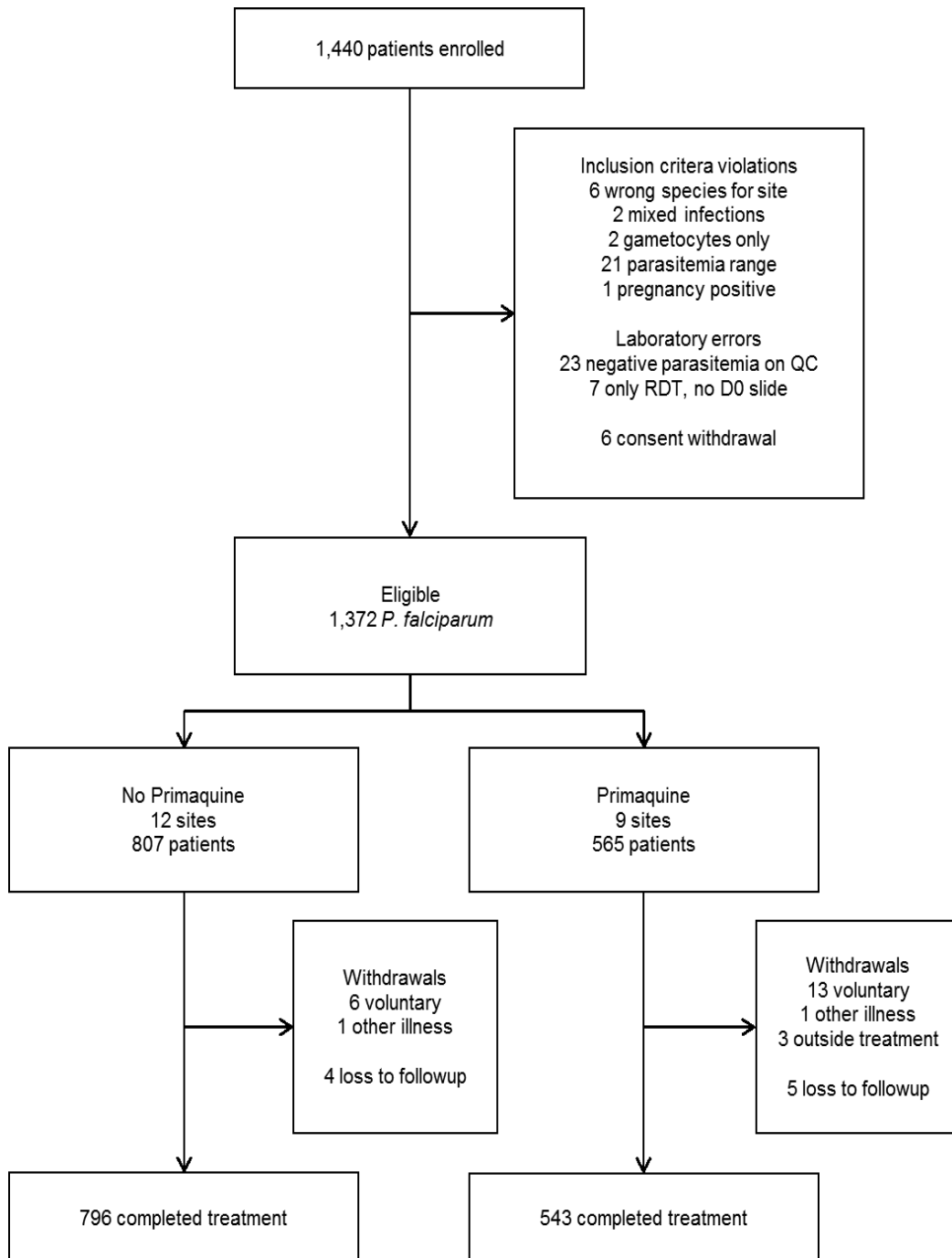
FIGURE 6.2. Prevalence of gametocytemia by treatment arm at each study visit among patients from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010
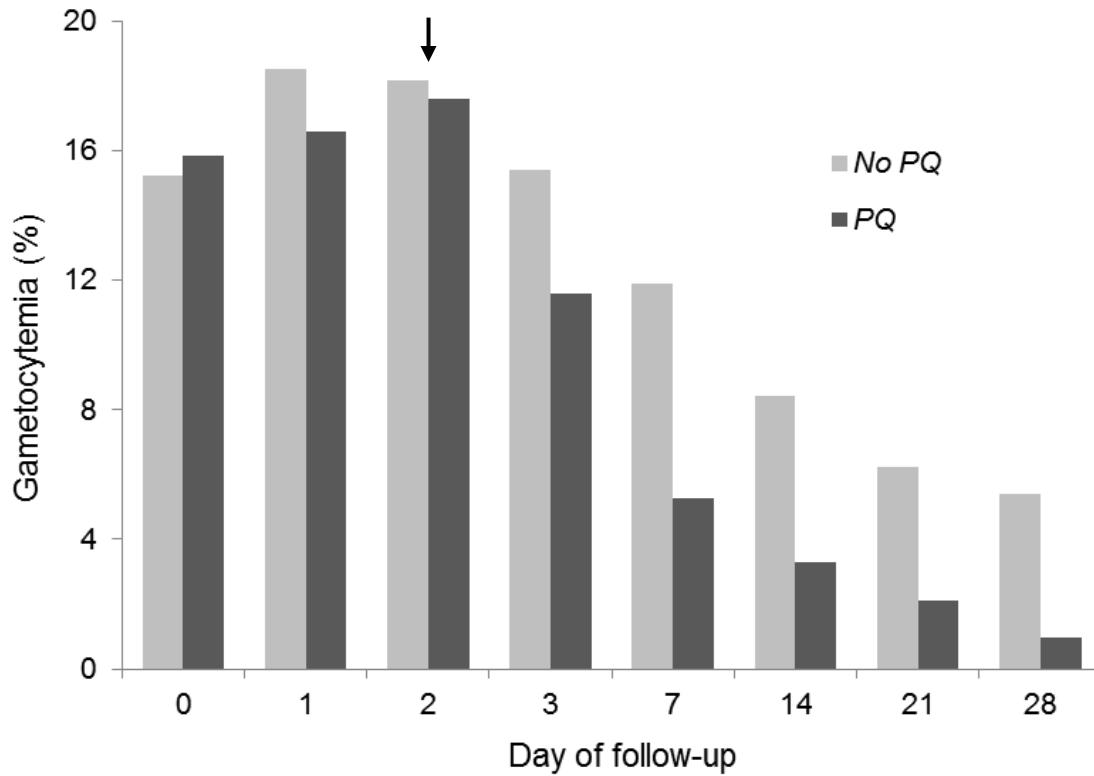
Abbreviations: PQ, primaquine; Arrow, administration of primaquine

FIGURE 6.3. Clearance of gametocytes by treatment arm among patients with pre-treatment gametocytemia from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010
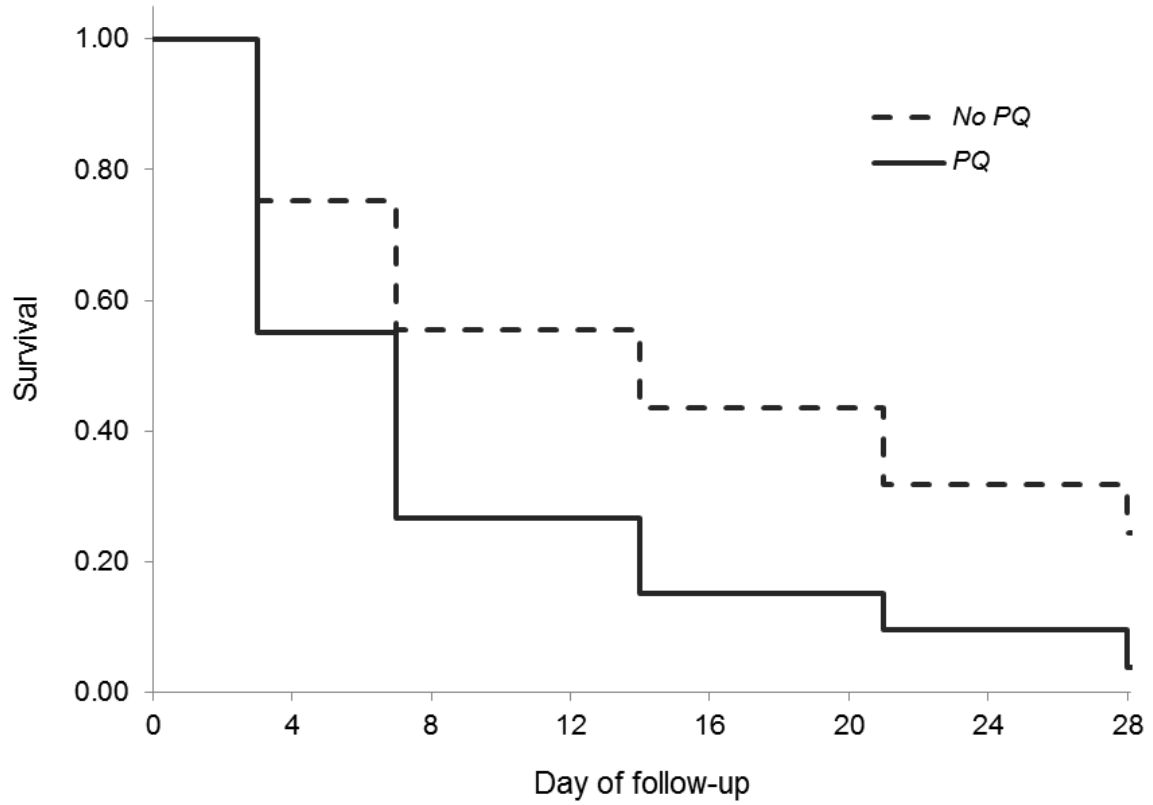
Abbreviations: PQ, primaquine

FIGURE 6.4. Area under the curve for the mean gametocyte density by treatment arm at each study visit among patients from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010
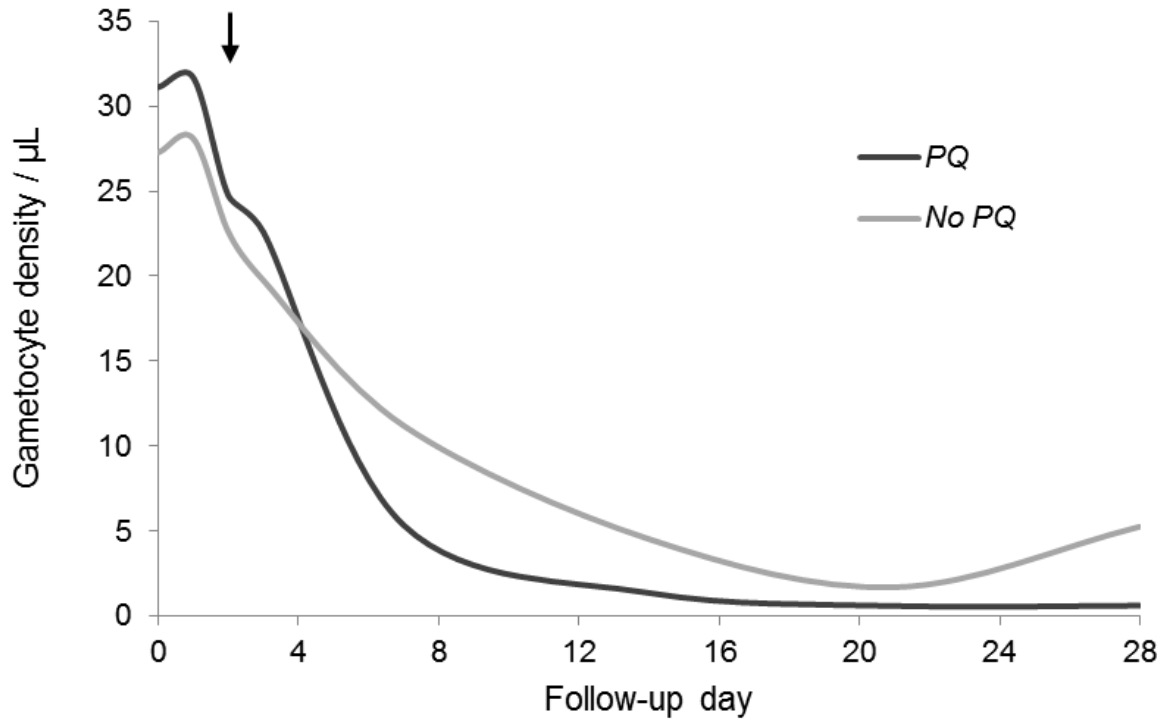
Abbreviations: PQ, primaquine; Arrow, administration of primaquine

CHAPTER SEVEN: DISCUSSION

Gametocytes are the sexual stage of *Plasmodia* life cycle which render malaria cases infectious to mosquitoes and propagate transmission. The proportion of *P. falciparum* malaria cases with gametocytemia and the duration and density of that gametocytemia are varied. In areas of low transmission, such as most of India, the number of infective hosts, as opposed to vectorial capacity, determines transmission. Thus, studies of the epidemiology of gametocytemia are needed to better define this key reservoir. Interventions for detecting and treating gametocytemia also differ from those used against asexual parasitemia. So an improved understanding of gametocytes opens the possibility of distinct transmission-blocking control strategies for the nation.

First, which case-patients carry gametocytes? Universal application of gametocytocidal interventions adds cost and may expose some individuals to potentially severe side effects. Targeting interventions by direct examination of gametocytes during routine microscopy can be difficult due to low density, poor training, and the heavy workload of the microscopist. Easily discerned risk factors associated with gametocytemia could help but these have not been assessed in Indian transmission settings where, in comparison to sub-Saharan Africa, natural immunity is low, many patients are adults, and the presence of competing malaria species may be an important determinant. Risk factors used in a clinical risk score could enable field-level screening.

Second, how should we treat gametocytemia? Artemisinin combination therapies (ACT), the first-line treatment in most countries including India, eliminate immature gametocytes but not mature gametocytes which may persist for up to one month post-treatment. The key operational question is whether a single dose of primaquine, which is inexpensive and effective against mature gametocytes, should be added to artemisinin combination therapies to reduce the potential post-treatment transmission of the infection. However, we need data regarding the safety or effectiveness of doing so. This dissertation aimed to describe the epidemiology of gametocytemia in India, including its prevalence, age-structure, and risk factors, as well as estimate the effect of primaquine in addition to an ACT for improving the control of malaria in the country by reducing potential transmission.

SUMMARY OF FINDINGS

In this dissertation we reported several findings which may help improve malaria control operations in India, and potentially elsewhere. In our first specific aim, we measured the prevalence of gametocytemia in India in patients recruited from the National Antimalarial Drug Monitoring System during 2009 to 2010. Gametocytemia which varied by region and age was highest in Western India, where transmission is low, and lower in the Central and Northeastern regions, where transmission is more intense. Gametocytemia was highest in children and decreased with age; however, when examining potential reservoir for transmission in the study population, adults constituted nearly half of the total indicating a substantial role in the spread of malaria. We found four easily screened risk factors associated with gametocytemia – age, region, gender, and previous antimalarial drug intake. While the predictive ability of the clinical algorithm to detect gametocytemia was low, using

this model we could eliminate gametocytocidal treatment in in 31% of patients while maintaining more than 90% sensitivity for detecting carriers in our study population.

In our second specific aim, we estimated the effect of adding primaquine to AS+SP for reducing the potential of post-treatment transmission of *P. falciparum* in India through a natural experiment of trials from the National Antimalarial Drug Monitoring System during 2009 to 2010. We found the gametocytocidal dose of primaquine increased the rate of gametocyte clearance in patients who were positive pre-treatment, decreased the circulation of gametocyte-weeks in all patients regardless of pre-treatment status, and shrunk the AUC of gametocyte density over time. The estimated effect of primaquine was robust to adjustment by area, as well as other sets of covariates. The estimated effect of primaquine, on gametocyte clearance or circulation, increased in sensitivity analysis of the exposure time assigned. No serious adverse events were reported among either group though the proportion of patients with vomiting was slightly higher among primaquine recipients.

PUBLIC HEALTH SIGNIFICANCE AND FUTURE DIRECTIONS

Specific Aim 1

Our findings in Aim 1 suggest that individual level targeting of gametocytocidal interventions may not be effective since the predictive power of our clinical algorithm was low even with the use of multiple risk factors. While the direct costs of using an algorithmic approach are non-existent, and any reduction of false-positives while minimizing false negatives would be beneficial compared to universal treatment, substantial indirect costs exist. Considerations of implementing any algorithm must account for the operational challenges in individual level targeting including the costs of training, the time required for patient assessment, and increased programme complexity. Population-based targeting may

also not be effective. Each age group contributed substantially to the potential reservoir for transmission suggesting that age-based targeting such as intervening on young or school-age children for example, may not provide sufficient reduction if adults were ignored. Similarly, the prevalence of gametocytemia was greater than 5% in all regions. However, in Western India, we observed 40% of patients with gametocytemia. Given the lower vectorial capacity in this region, gametocytocidal interventions may have greater proportional impact on transmission here. Overall, our results suggest the need for the universal application, if any, of gametocytocidal interventions among confirmed malaria patients. Future research on gametocytemia could try to measure the asymptomatic reservoir and conduct longitudinal assessment. Finally, in Thailand the malaria control programme records the prevalence of gametocytemia, which arises three to four days after patency, as an indicator for monitoring access to care. Patients treated early in their disease course are less likely to carry gametocytes. Given the wide network of microscopy clinics, India could also make use of gametocytemia as a monitoring indicator for treatment delay.

Specific Aim 2

Our findings in Aim 2 demonstrated the gametocytocidal effectiveness of primaquine when added to AS+SP compared to AS+SP alone. The administration of single-dose primaquine along with artemisinin combination therapy in India should be considered to improve malaria control. Several avenues of future operational research are needed for optimizing the adjunctive use of primaquine. While primaquine certainly works, several operational questions remain regarding the optimal dose, time of administration, adherence, and safety. The current dose of primaquine was determined through limited experiments in the 1960s (66,67). Lower, or higher, doses of primaquine may be equally, or more, effective

– Thailand, for example, uses a 30mg adult dose of primaquine for gametocytocidal therapy (60). The day of administration of primaquine varies from day zero to day two with little evidence available to guide policy. For day zero, non-fixed-dose therapies, such as AS+SP, have a larger pill burden on day zero but on the other hand treatment can be observed. For day three, with treatment occurring, most likely, after the completion of asexual parasite clearance, more mature gametocytes can be exposed to the drug since the window of exposure is short. Modeling studies have suggested later administration one week after treatment may have the most transmission reducing effect (140). However, such a regimen could be operationally difficult.

Primaquine also poses a safety risk given the risk of hemolysis in individuals with G6PD deficiency (63). The prevalence of G6PD deficiency in India is not well mapped but may be present in up to 10% of some tribal groups (141), populations in which the malaria burden is also concentrated (142). However, most severe events have been associated with the longer primaquine dosing used for anti-relapse treatment in *P. vivax* and not the gametocytocidal single-dose. Recent research suggests hemolysis may occur in non-G6PD individuals as well though severe anemia was rare (71). In the previous primaquine trials, hemoglobin concentrations among exposed patients were 5% lower at day seven (recovered by one month) or the mean increase over the study was 0.3g/dL less compared to patients who only received artemisinin combination therapy (47,75). The only adverse effect attributable to primaquine in any study was increased abdominal pain (75,76). However, hemolysis and side-effects will vary by population and in the context of already vulnerable populations (74) more assessments, particularly post-marketing pharmacovigilance, are needed.

CONCLUSIONS

The need to reduce the reservoir of transmission for malaria control was recognized more than 100 years ago (143). In modern malaria control programs the opportunity to do so may be greater than ever before. Case management is now a central, and perhaps the primary, component of many control efforts while in the eradication era it was an after-thought to massive operations to reduce vector longevity. There are several reasons for the change in outlook. We now have many new and more effective treatments, more equipment (rapid diagnostic tests) and more infrastructure (better health systems) for diagnosis, but we also have fundamentally different goals. Reducing the morbidity and mortality burden of the disease, through access to care, is considered feasible, sustainable, and desirable by planners and communities (144). Thus, the opportunity to simultaneously reduce transmission, while enhancing case management, is attractive given its alignment with overall strategy and the very low direct and indirect costs of doing so (for single dose primaquine). This dissertation adds evidence about whom to target for transmission reduction interventions and how effective primaquine may be in the current context of ACT treatment. Finally, it would be remiss to fail to note that the appropriate use of new indicators, interventions, and operational strategies ultimately rely on stronger health systems to enable their effective deployment and maintain the resultant gains made in public health (145,146).

APPENDIX A: CASE RECORD FORM

Case Record Form used in Therapeutic Efficacy Trials through the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

CASE RECORD FORM

STUDY SITE	Health Facility's Name:		Town:		District/province:	
PATIENT	Identity Number:	Full Name:		Age (years):	Sex (M/F):	Weight (kg):	Height (cm):
	Name of guardian:		Contact home address:			

ANTIMALARIAL DRUG	Drug name:	Manufacturer:	Batch number:	Expiry date:	Total dose (mg base):
	Previous intake (Y/N/Unknown):	Drug:	Date:	Total dose (mg):	Urinary test (name):	Result

DAY	DAY 0	DAY 1	DAY 2	DAY 3	DAY 7	DAY 14	DAY 21	DAY 28	Unscheduled Day
Date									
Danger signs (Y/N)									
History of fever Last 24 hrs (Y/N)									
Axillary Temperature (°C)									
Asexual parasite+Sexual count (per µl)									
Treatment No of tablets									
Concomitant Treatment									
Possible drug side-effects of antimalarials									
Observations									

Overall assessment:	Early treatment failure (ETF)	Late clinical failure (LCF)	Late parasitological failure (LPF)	Adequate clinical and parasitological response (ACPR)	Withdrawn (WTH)	Loss to follow-up (LOSS)
Day of assessment:					Reason for WTH	

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APPENDIX B: NEW SYSTEM FOR MONITORING ANTIMALARIAL DRUG RESISTANCE IN INDIA

Nationwide Sentinel Site System For Monitoring Antimalarial Drug Resistance In India: Outcomes And Risks For Treatment Failure, 2009-2010

by

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Abstract

Background

Study protocols for monitoring the efficacy of antimalarial drugs are well standardized; however, routine systems for selecting sites and conducting trials are not. The recently created National Antimalarial Drug Resistance Monitoring System of India is characterized by alternating sentinel sites for therapeutic efficacy trials, centralized data management, quality control checks, genotyping of molecular markers, and the integration of supplementary studies. We aimed to describe the performance of the new system, measure the efficacy of the first-line malaria treatments, and determine risk factors associated with treatment failure.

Methods

During 2009 and 2010, we recruited patients in prospective single arm, open label trials with 28 days of follow-up. In 22 studies, patients received artesunate plus sulfadoxine-pyrimethamine (AS+SP) for the treatment of *Plasmodium falciparum* and in 3 studies, patients received chloroquine for the treatment of *P. vivax* according to national policy. We genotyped four *dhfr* and three *dhps* codons of 20% of the *P. falciparum* isolates including all

treatment failures. We used multivariate exponential risk regression to determine the association of clinical and demographic factors with treatment failure.

Results

We enrolled 1,664 patients in 25 therapeutic efficacy studies across India. The overall crude and PCR-corrected Kaplan-Meier efficacies for AS+SP were 98.6% (95%CI: 97.8, 99.1) and 98.8% (95%CI: 98.1, 99.3) respectively. The majority of *P. falciparum* patients cleared parasitaemia within 24 hours. Four of six patients who did not clear parasitemia within 72 hours failed treatment. Genotyping revealed a high prevalence of double mutants in *dhfr* (68.4%, n=234) and a few triple and quadruple mutants (3.2%, n=11) while mutations in *dhps* were rare (2.3%, n=6). An administered dose of artesunate <3mg/kg, age less than five years, fever at the time of enrollment, and a parasite density less than 5,000/μL increased the risk of treatment failure. Chloroquine remained 100% efficacious in the treatment of *P. vivax* and the majority of patients cleared parasitemia prior to 48 hours. Few adverse events to either therapy were reported, the most common being vomiting (n=47).

Conclusions

First-line drugs for the treatment of malaria in India remain safe and efficacious. The new monitoring system which coordinated resources provided wide coverage which detected two sites for additional monitoring. Pooled analysis led to the identification of risk factors which indicate the need for future studies particularly in the pediatric population and trials comparing age-based and weight-based dosing.

Introduction

The global control of malaria remains a major challenge and is aggravated by parasite resistance to antimalarial drugs. *Plasmodium falciparum* rapidly develops resistance to new

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therapies and the presence of multi-drug resistant, now including artemisinin, strains in Southeast Asia is alarming (147). *Plasmodium vivax* develops chloroquine (CQ) resistance over time, is innately less susceptible to anti-folate compounds, and the sensitivity of its dormant stages to primaquine, the only anti-relapse therapy, is difficult to assess (148). The long-term public health response to antimalarial drug resistance requires reducing drug pressure through rational treatment and creating a pipeline of alternative drugs. Its routine management however, centers on monitoring the efficacy of current therapies and selecting appropriate treatments based on that evidence. Several monitoring techniques are used to inform policy-making: *in vitro* drug sensitivity tests, pharmacokinetic studies, molecular markers of drug resistance, and *in vivo* therapeutic efficacy trials (149).

The Republic of India has conducted extensive monitoring of antimalarial drug resistance over many decades. Chloroquine resistant *P. falciparum* was first reported in Karbi-Anglong district, near the Indo-Myanmar border, in 1973 and subsequent studies confirmed its presence throughout the Northeast (150). CQ resistant *P. vivax* was unknown in India until 1995 when Garg et al. reported two cases from Mumbai who remained smear positive despite adequate blood concentration of drug (151). Similar case reports from Northern and Eastern India have seen emerged (152,153). In 1978, the National Malaria Eradication Programme (now the National Vector Borne Disease Control Programme or NVBDCP) created six regional monitoring teams to routinely conduct studies. In addition, the Malaria Research Centre (now the National Institute of Malaria Research or NIMR) and other organizations supported a wide range of monitoring efforts. Between 1978 and 2007, *in vivo* trials of the first-line and second-line *P. falciparum* treatments, CQ and sulfadoxine-pyrimethamine (SP), alone consisted of 380 studies with 18,944 patients (107). The median proportion of treatment failures with SP had increased from 7.7% during 1984-96 to 25.9%

during 1997-2007 in 28 days of follow-up (107). Indian isolates also demonstrated widespread mutations in the drug targets dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*), including quintuple mutants in some areas (154). As the efficacy and life-span of artemisinin combination therapies depend largely on the partner drug, pre-existing resistance to SP creates the possibility of rapid development of resistance to the new combination. Attributing study results to individual components of the combination therapy is difficult, and assessing the efficacy of the partner drug alone is no longer ethical. In 2005, artesunate plus sulfadoxine-pyrimethamine (AS+SP) replaced SP in the national drug policy and is now the universal first-line treatment for *P. falciparum* in India. While the efficacy of AS+SP was high (0% median failure, 4% max) in nine 28 day follow-up studies from 2005-2007, the monitored population was limited given the size of the country (149).

Addressing these challenges necessitated the creation of a joint NVBDCP-NIMR surveillance system with several innovations: 1) alternating sentinel sites to provide both longitudinal trends along with widespread coverage, 2) routine *P. vivax* trials to track the emergence of CQ resistance, 3) central quality control of slide results and data analysis, 4) genotyping to differentiate recrudescent samples from re-infections, 5) simultaneous measurement of molecular markers of drug resistance, and 6) integration of supplementary studies such as pharmacokinetics and residual drug levels. We collected and analyzed data from the first two years of operating the new system to describe treatment outcomes across India and determine the factors associated with treatment failure. First, we estimated the efficacy of AS+SP and CQ for *P. falciparum* and *P. vivax* respectively. Second, we measured molecular markers of SP resistance to independently assess the partner drug. Finally, we determined the clinical, demographic, and parasite-related predictors of treatment outcomes.

Methods

Study sites and population

The NVBDCP reported 1.6 million malaria cases in 2009 and 2010 through its extensive surveillance system which conducts more than 100 million screenings for malaria each year (126). NIMR and NDVBDCP selected 24 districts (third level administrative unit, approximate population 1 to 4 million) and one city across 13 states and one union territory of India respectively (Figure 1) during annual reviews of the antimalarial drug resistance data. Sites were purposively selected to provide a representative sample of varying transmission intensity, malaria ecotype, and geographic region (Figure 1). We classified sites into area categories reflecting malaria ecotypes: western – unstable rural or urban transmission driven by *Anopheles culicifacies* and *A. stephensi*, central – stable forest-related transmission driven by *A. fluviatilis*, and northeastern – stable de-forestation and forest-related transmission driven by *A. minimus* and *A. dirus* (82). Trials were open label, single arm prospective studies using the 2009 World Health Organization (WHO) protocol for therapeutic efficacy trials with a target sample size of more than 50 patients (117). We included consenting patients with *P. falciparum* (asexual parasites 500-100,000/μl) or *P. vivax* (asexual parasites >250/μl) monoinfection and current fever (axillary temperature ≥ 37.5^{0}C) or a history of fever in the preceding 24 hours. We excluded pregnant or lactating women, children weighing less than five kilograms, and patients with signs of severe malaria.

Data collection, treatment, and follow-up

At enrollment, we recorded a complete medical history of presenting symptoms, current medications, and previous antimalarial drug use for each patient. We tested female patients for pregnancy and collected thick and thin blood smears and dried blood spots on

filter paper. We treated patients with age-specific treatment according to the national drug policy (14). Patients with *P. falciparum* received AS+SP (artesunate 4mg/kg for 3 days plus SP 25/1.25mg/kg single dose) and those with *P. vivax* received CQ (25mg/kg over 3 days) as well as primaquine (0.25mg/kg for 14 days) after completing follow-up. We observed treatment and used quality-assured drugs through the state government supply which consisted of the following manufacturers (number of sites in brackets): AS+SP - IPCA (11), Medicamen (5), Zydus (2), Hindustan Antibiotics, Unicure, Corona Remedies, Leonate, and CQ – IDPL (2), Troikaa. All drugs were used within their expiry period and batch numbers are available upon request. Patients who vomited the dose within 30 minutes were re-treated and those with repeated emesis were withdrawn from the study and treated as severe malaria. We followed patients on days 1, 2, 3, 7, 14, 21, and 28 in the clinic or traced them to their residence or place of work. We also advised patients to return at any time if they experienced fever or other symptoms. Patients who did not complete treatment were withdrawn from the study. At each follow-up visit, we performed a physical examination and collected blood smears and dried blood spots. At the end of follow-up patients received Rs 500 for lost wages.

Laboratory procedures

We collected thick and thin blood films on the same slide and stained them with Giemsa. Parasites were counted on thick films against 200 white blood cells (WBC) using 100X light microscopy by technicians with more than ten years of experience. We calculated assuming 8,000 WBC/μl of blood. Slides were declared negative if no parasites were detected in 100 high-power fields. Expert microscopists cross-checked all failure samples as well as a 10% random sample of positive and negative slides from each site at the central

quality control unit at the NIMR headquarters in New Delhi. We analyzed paired blood samples from day zero and the day of recrudescence, if any, by Polymerase Chain Reaction (PCR) to rule out re-infections. Parasite genomic DNA was isolated from filter paper blood spots using the QIAamp DNA blood mini kit and 2μl of eluted DNA were used for the primary PCR assay and 1-2μl of primary PCR product was used for nested PCR. The markers *msp-1*, *msp-2*, and *glurp* were analyzed in paired samples to distinguish recrudescence from new infections using previously published methods (155). In addition, we genotyped the molecular markers for SP resistance in all day zero samples from patients who failed treatment along with 25% of other samples selected at random. We conducted *P. falciparum dhfr* and *dhps* amplification using primers described earlier (156). A mutation-specific nested PCR was performed with the primary product to detect *dhfr* codons 51, 59, 108, 164 and *dhps* codons 436, 437, 540.

Trial endpoints

Primary study endpoints were classified according to standard criteria defined in the current WHO guidelines (13). Briefly, for endpoints included adequate clinical and parasitological response (ACPR), early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), loss to follow-up (LFU), and voluntary/involuntary withdrawal or exclusion due to protocol violation (WTH). Treatment failures (ETF, LCF, and LPF) were then classified as recrudescence or re-infection according to PCR results. We examined parasite clearance interval (<24hr, 24-48hrs, 48-72hrs, >72hrs), taken the first follow-up day with two consecutive days of negative blood smear prior to day three or the first negative blood smear on day three or day seven, as a secondary endpoint. For therapies with high efficacy, as was expected for AS+SP, parasite clearance interval can provide

evidence of emerging resistance as its prolongation in the population can precede the frequent appearance of treatment failure. We used binary cut-offs of >72hrs clearance as the endpoint for risk factor analysis.

Data analysis

We selected patient and demographic factors which could affect treatment failure and defined categories of age and parasite density with cut-offs used in the literature. Season was classified by month of enrollment: monsoon – June-August, post-monsoon – September-November, and winter – December and January. We defined anti-folate molecular markers as the sum of total *dhfr* plus *dhps* mutations detected. If *dhps* codon data were not available because of a failure to amplify, *dhfr* data alone were used. The use of age-based dosing, rather than weight-based dosing, means the administered drug dose may not have matched the target dose. We calculated the administered dose for *P. falciparum* patients by dividing the age-wise dose by the patient's weight and categorized it as follows: ≥87.5% of target dose, 75-87.5% of target dose, and <75% of target dose. For artesunate and SP the respective cut-offs were ≥3.5mg/kg, 3-3.5 mg/kg, <3 mg/kg and ≥22 mg/kg, 19-22 mg/kg, and <19 mg/kg.

We described the basic characteristics of patients as well as the primary and secondary outcomes by the species of infection. To estimate drug efficacy, we conducted per protocol analysis and modified per protocol using Kaplan-Meier survival analysis, with and without PCR-correction, of treatment failure and parasite clearance. We used log-risk models to calculate the univariate and multivariate associations between risk factors and endpoints. For multivariable analysis we constructed full models with age, area, gender, fever at enrollment, parasite density at enrollment, anti-folate mutations, administered artesunate

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dose, and interaction between age and administered artesunate, age and parasite density, and anti-folate mutations and parasite density. We used a backwards elimination strategy with a cut-off of p<0.10 for predictors and p<0.15 for interaction terms. Multi-level categorical variables were reduced to dichotomous variables to preserve power prior to their removal from models. To account for the clustering of data by study site, we used clustered robust standard error in both multivariate models to adjust the precision of estimated risks. STATA (version 10) was used for all analyses.

Human subjects protection

We obtained informed, written consent from patients or their guardian. The trials were approved by the Ministry of Health and Family Welfare, Government of India as well as the ethics committee of National Institute of Malaria Research.

Results

Patient enrollment and characteristics

A total of 1,733 patients were enrolled during the first and second years from 25 sites (Figure 2). 895 patients were enrolled between June 2009 to January 2010 at 13 sites and 838 patients were enrolled between June to December 2010 at 12 sites. Out of 1,664 eligible patients, 1,394 *P. falciparum* and 185 *P. vivax* patients completed follow-up until day 28 (94.8%). Most trial patients were male, adults, febrile at the time of enrollment, from Central India, and recruited in the monsoon (*P. vivax*) or post-monsoon (*P. falciparum*) season (Table 1). A history of fever was near universal while the intake of antimalarial drugs in the previous week was rare. The geometric mean parasitemia was 7,975 parasites/μl (range: 560, 99,707) for *P. falciparum* and 7,133 parasites/μl (range 378, 123,317) for *P. vivax*.

P. falciparum

Primary and secondary outcomes

1,394 patients completed the study follow-up (Table 2). The majority of patients demonstrated clinical and parasitological cure and few withdrew or were lost to follow-up. Out of 27 cases of treatment failure, there were 17 true failures (63%) across 10 trial sites. The other treatment failures were *P. vivax* infections or *P. falciparum* re-infections (both on day 28) while one case was untypeable (day 28 failure). Two early treatment failures occurred in Betul with parasitemia and fever on day three and one in Angul with day three parasitemia more than 25% of day zero parasitemia. In Gadcharoli, five patients failed treatment on day 7. The crude and PCR-corrected per protocol Kaplan-Meier survival estimates were 98.6% (95%CI: 97.8, 99.1) and 98.8% (95%CI: 98.1, 99.3) respectively (Figure 3A). Among sites, the survival estimates ranged from 93.8% to 100% (Table S1). *P. falciparum* patients treated with AS+SP cleared parasitemia rapidly with 62% aparasitemic within 24 hours of administering the drug (Table 3B). Of the six patients who did not clear parasitemia within 72 hours, four (67%) recrudesced (including three early treatment failures) while the rest achieved cure. Vivax and falciparum re-infections occurred among patients who cleared parasitemia prior to day three. For the categories <24hours, 24-48hours, and 48-72 hours the proportion who recrudesced were 0.7% (6/873), 1.3% (5/384), and 1.3% (2/154) respectively. Four patients (81%) of the six with clearance greater than 72 hours were from Betul. AS+SP treatment was well tolerated. Adverse events reported during follow-up included vomiting (46), fever (3), and jaundice (1).

Molecular markers of drug resistance

342 of 373 isolates genotyped for *dhfr* successfully amplified. Single (n=65) and double mutations (n=234) were common. The most frequent haplotypes included the double mutant 108/59, then single mutant 108 (Table S3). Only 10 and 1 isolates were triple or quadruple mutants respectively (3.2%). Typing of *dhps* proved difficult and only 261 of 373 isolates were successfully amplified. Among these isolates, mutations in *dhps* were rare (2.3%) with five single mutations and one double mutation. Mutations in codon 437 were the most frequent. Among the 17 PCR-corrected treatment failures, one sample did not amplify, 12 were *dhfr* double mutants, 4 were single mutants, and all were wild-type for *dhps*.

Predictors of treatment failure and parasite clearance interval

For PCR-corrected treatment failure, younger age category (<5 years) relative to adults 15 years or older, and low administered dose of artesunate (<3mg/kg, 3-3.5mg/kg) relative to 3.5mg/kg or more were associated with higher risk (Table S2). In multivariate analysis, age less than five years, fever at the time of presentation, and an administered artesunate doses less than 3.5mg/kg and less than 3mg/kg were associated with treatment failure (Table 3). For administered artesunate dose the relative risk of failure increased with as the dose category decreased. A parasite density of 5,000/μL or more was associated with a lower risk of treatment failure. We did not detect any association between increased mutations (beyond 1-2) in *dhfr* and *dhps* and treatment failure of AS+SP. For parasite clearance interval of 72 hours or more, there were insufficient observations to determine its predictors.

P. vivax

Three trials of CQ in 185 patients demonstrated 100% efficacy of the drug. However, compared to *P. falciparum* patients, a higher proportion of *P. vivax* patients, many of whom were residents of urban slums, withdrew or were lost to follow-up (Table 1). Most patients (56.2%) cleared parasitemia by 48 hours (Figure 3B). In six patients parasitemia persisted after 72 hours but without any symptoms and cleared by day seven of follow-up. CQ treatment was well tolerated. We observed one event of each of the following: gastritis, stomatitis, and vomiting.

Discussion

The National Antimalarial Drug Resistance Monitoring System recruited 1,733 patients and completed therapeutic efficacy trials in 25 sites across India during its first two years. The results suggest first-line therapies for *P. falciparum* and *P. vivax* recommended by the national antimalarial drug policy, AS+SP and CQ, remain efficacious.

P. falciparum

The 28 day efficacy of AS+SP for the treatment of *P. falciparum* was more than 98%. While treatment failures and isolates with prolonged clearance were few, those identified were clustered among a few sites. Surprisingly, these did not include Northeastern India where the highest rates of SP monotherapy failure have been reported (157). The association of parasite clearance interval greater than 72 hours with treatment failure (4/6) suggests the measurement of clearance may be an important indicator in the age of combination therapy. The apparent clustering validates the design of the new monitoring system – to provide wide coverage to detect such variation and longitudinal studies to follow-up on emerging trends.

Besides recrudescent infections, *P. falciparum* re-infection was rare. While the re-infection rate may be a function of the length of the follow-up period, it also depends on the intensity of transmission which is generally lower in India compared to many other regions.

Consistent with previous studies, most isolates (87.1%) were partially pyrimethamine resistant with single (S108N) and double mutations (S108N/C59R). We detected a low prevalence of triple and quadruple mutants though (3.2%). *dhfr* single and double mutations increase the IC50 of pyrimethamine ten-fold while the triple mutation increases the IC50 by 1,000 times. Seven isolates possessed the I164L mutation associated with high-level resistance (158). Many samples failed *dhps* amplification (27.6%) though the failure rate was similar to that previously published using the same protocol which generates large amplicons (154). Still, single or double mutation among the successfully genotyped isolates was low (2.3%) assuming that mutations were not the cause of non-amplification. Monitoring step-wise increases in both markers will alert us to increasing resistance independent of clinical response.

Treatment failure reflects a combination of drug resistance, host immunity, and pharmacokinetics. Predictors of treatment failure included elements of the latter two in this study. Younger age, fever at the time of presentation, and a lower parasite density, all potential markers of lower immunity, were also associated with one or both outcomes. Finally, a lower administered dose of artesunate was associated with treatment failure in a dose-response manner. Overall, 8.8% of the patient population were administered an artesunate dose between 3-3.5mg/kg and 1.9% received less than 3mg/kg. In comparison, 1.3% of received an SP dose between 19-22mg/kg and 0.3% received less than 19mg/kg. The blister pack dose of AS+SP (200mg) assumes a 50kg adult body weight to achieve the target artesunate dose. However, the SP dose (1500mg) assumes 60kg adult body weight to achieve

the target and is therefore less frequently underdosed. The relationship between administered dose and blood levels of drug however, is complex.

P. vivax

In spite of sporadic case reports of CQ-resistant vivax in India, we did not detect any treatment failures during systematic trials. We did not obtain blood levels of CQ because there were no treatment failures. While trials were, thus far, located in southern India, the areas were major urban centers and many patients were migrant workers from all over the country. More vivax malaria studies in other locations are needed though adding sites will result in a longer period between repeating therapeutic efficacy trials at each. Given the relative slower pace of drug resistance development compared to *P. falciparum* this may be acceptable. A critical component of vivax malaria therapy is primaquine treatment. However, no standard protocols for evaluating the therapeutic efficacy, alone or in combination with CQ, exist. Another remaining challenge is the treatment of mixed infections. Due to their exclusion, we do not know the efficacy of AS+SP for treating vivax malaria which is the current protocol for such cases. Recent reports across Southeast Asia have described a high incidence of vivax malaria following the treatment of *P. falciparum*, presumably from liver-stage relapses (159). We observed seven episodes of *P. vivax* during the follow-up of our *P. falciparum* patients. Six of the post-treatment *P. vivax* cases were from two sites, Angul and Dhenkenal in Orissa, representing a relapse rate of 5.2% at each site.

Sentinel site monitoring

We designed and implemented a new national monitoring system for India. The benefits included high data quality, national representation of different malaria ecotypes,

coordinated use of resources, and pooled data analysis. While the number of sites exceed the minimum recommended by WHO, even more may be required in a country as large as India. The ability to alternate sentinel sites as planned will provide longitudinal trends as well. We did face several challenges. While overall coordination in the joint NVBDCP-NIMR system was satisfactory, we encountered occasional administrative delays which led to delayed recruitment at some sites. Other difficulties encountered were the central procurement of some reagents, e.g. pregnancy testing supplies, sample transport for quality control to New Delhi, and local data entry in a digital format. These issues were to be expected as with any new program and the coordination was reviewed and addressed. Using data and biological samples collected through the system, NIMR investigators have initiated several supplementary work including 1) the prevalence of residual antimalarial drug levels at enrollment and its association with molecular markers and outcomes, 2) pharmacokinetics of SP in treatment failure samples, and 3) pharmacovigilance of antimalarials used in the country.

Limitations

Our study had several limitations. The 28 day follow-up, although recommended by WHO, could be longer. If the drug efficacy is high then any treatment failures are likely to occur late, especially in partner drugs with longer half-lives such as SP. Thus, we implemented a 42 day follow-up for three studies in 2011 and for all upcoming studies in 2012 to detect later failures. Few treatment failures limited the power to detect its predictors. Next, monitoring of parasitemia every 24hrs is not frequent enough for accurate estimation of mean clearance times or stage-specific clearance (160). However, examining the proportion of aparastiemic patients by day intervals can still provide useful trends albeit with less power

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than a continuous measure. The cost and staff workload of nested-PCR assays to genotype molecular markers limited the number of samples assessed. Finally, while we describe the outputs of the new system, we have not calculated the costs of the new system which is complex as it uses resources across different institutions and between state and central levels.

Conclusions

AS+SP is a safe and effective for the treatment of uncomplicated falciparum malaria in India. CQ remains a safe and effective for the treatment of uncomplicated vivax malaria. Based on the two year implementation of the new system and the findings from initial studies, we recommend the following: 1) maintain sentinel site based antimalarial drug monitoring, 2) validate parasite clearance greater than 72 hours and subsequent treatment failure particularly in Betul, 3) switch to high-throughput technology, such as real-time PCR, for molecular work to enable the genotyping of more isolates, 4) conduct supplementary studies focused on pediatric populations to optimize treatment guidelines for this age group given their higher risk of failure, and 5) compare age-wise dosing with weight-based dosing, or evaluate increased blister pack doses based on higher average body weights. Endemic countries can design strategies to systematically monitor the changing threat of antimalarial drug resistance.

Figures and Tables

FIGURE B.1. Sentinel sites of the National Antimalarial Drug Resistance Monitoring System by year, parasite species, and state (district), India, 2009-2010

STUDY SITES 2009

1. Assam (Nagaon)
2. Chhattisgarh (Kanker)
3. Dadra Nagar Heveli UT (Silvassa)
4. Jharkhand (Ranchi)
5. Jharkhand (Simdega)
6. Madhya Pradesh (Chhindwara)
7. Maharashtra (Gadchiroli)
8. Orissa (Dhenkanal)
9. Orissa (Sambalpur)
10. Rajasthan (Baran)
11. West Bengal (Jalpaiguri)
12. Goa (North Goa)
13. Karnataka (Mangalore)

STUDY SITES 2010

14. Andhra Pradesh (Visakhapatnam)
15. Chhattisgarh (Bilaspur)
16. Gujarat (Surat)
17. Jharkhand (Dumka)
18. Jharkhand (Latehar)
19. Karnataka (Gulbarga)
20. Maharashtra (Mumbai)
21. Madhya Pradesh (Betul)
22. Meghalaya (West Garo Hills)
23. Orissa (Angul)
24. Rajasthan (Pratapgarh)
25. West Bengal (Kolkata)

Pf (AS+SP)

Pv (CQ)

FIGURE B.2. Patient cohort from the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

1,733 patients enrolled

Inclusion critera violations
6 wrong species
2 mixed infections
2 gametocytes only
22 parasitemia range
1 pregnancy positive

Laboratory error
23 negative parasitemia on QC
7 only RDT, no D0 slide

6 consent withdrawal

Eligible
1,454 *P. falciparum*
210 *P. vivax*

Withdrawals
33 voluntary
4 other illness
3 outside treatment

45 loss to followup

Completed protocol
1,394 *P. falciparum*
185 *P. vivax*

Failures
3 ETF
17 LPF
7 LCF

Successful treatment
1,367 *P. falciparum*
185 *P. vivax*

17 Recrudescent
2 Re-infections
1 Undetermined

7 Vivax infections

FIGURE B.3A. Kaplan-Meir survival of crude and PCR-corrected outcomes for *P. falciparum* in patients among eligible patients in studies of the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010
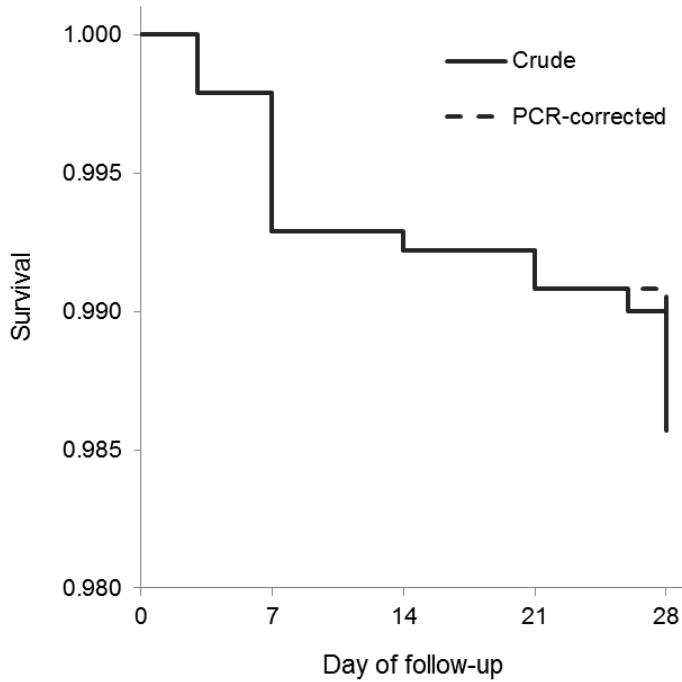
FIGURE B.3B. Kaplan-Meir survival of parasite clearance for *P. falciparum* and *P. vivax* by 24 hour intervals among eligible patients in studies of the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010
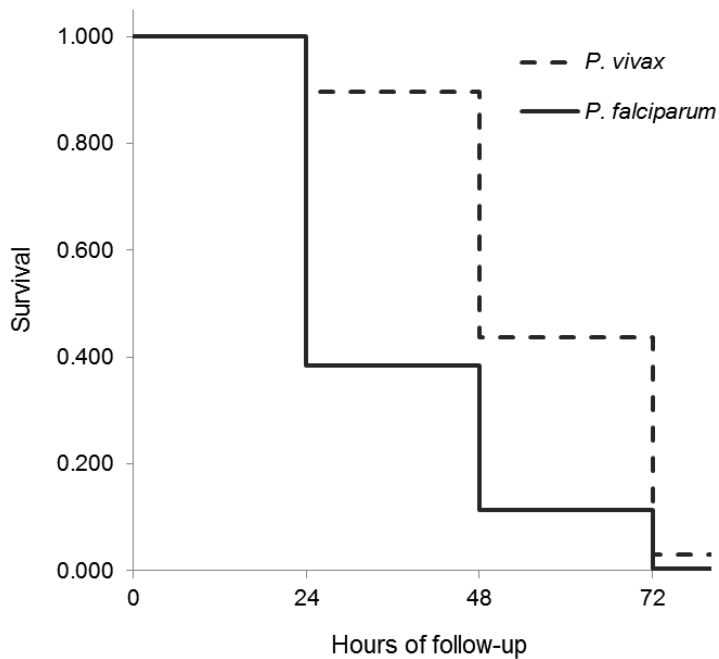
TABLE B.1. Clinical and demographic characteristics of eligible patients in studies of the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

Characteristic	Value	*P. falciparum* n	*P. falciparum* %	*P. vivax* n	*P. vivax* %
Area	Central	829	57.0	--	
	Western	391	26.9	55	26.2
	Northeast	234	16.1	--	
	Southern	--		155	73.8
Season	Monsoon	415	28.5	115	54.8
	Post-monsoon	645	44.4	95	45.2
	Winter	394	27.1	--	
Sex	Male	835	57.4	172	81.9
	Female	619	42.6	38	18.1
Age	<1	2	0.1	--	
(years)	1-4	120	8.3	2	1.0
	5-9	290	19.9	14	6.7
	10-14	252	17.3	18	8.6
	15-49	689	47.5	154	73.3
	≥50	101	7.0	22	10.5
Parasite count	<1,000	14	1.0	10	4.8
(/ µL)	1,000-5,000	548	37.7	69	32.9
	5,000-10,000	251	17.3	42	20.0
	10,000-50,000	526	36.2	83	39.5
	≥50,000	115	7.9	6	2.9
Febrile	No	451	31.0	63	30.1
(≥37.5°C)	Yes	1,003	69.0	146	69.0
History of fever	No	31	2.1	4	1.9
	Yes	1,423	97.9	206	98.1
Previous					
antimalarial	No	1,428	98.2	207	98.2
drug intake*	Yes	9	0.6	0	0.0
	Unknown	17	1.2	3	1.4

*missing from one site (n=25)

TABLE B.2. Primary and secondary outcomes over 28 days of follow-up among eligible patients in studies of the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

	Outcome	*P. falciparum*		*P. vivax*	
		n	%	n	%
Primary classification	Adequate clinical and parasitological response	1,367	94.0	185	88.1
	Early treatment failure	3	0.2	0	0.0
	Late clinical failure	7	0.5	0	0.0
	Late parasitological failure	17	1.2	0	0.0
	Lost to follow-up	33	2.3	12	5.7
	Withdrawal after day 0	27	1.9	13	6.2
PCR-corrected results	Recrudescent	17	63.0	--	
	Reinfection	2	7.4	--	
	Other species	7	25.9	--	
	Unknown	1	3.7	--	
Parasite clearance	≤ 24 hrs	873	61.6	20	10.4
	24 - 48 hrs	384	27.1	88	45.8
	48 - 72 hrs	154	10.9	78	40.6
	> 72 hrs	6	0.4	6	3.1

TABLE B.2S. Risk of *P. falciparum* treatment failure among patients in studies of the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

Predictor	Value	Fail	ACPR	Risk	RR	95%CI
Area	Central	10	786	0.013	--	
	Western	5	362	0.014	1.07	0.39, 2.93
	Northeast	2	229	0.009	0.52	0.07, 4.03
Age	< 5	5	112	0.043	4.62	1.49, 14.3
(years)	5 - 15	5	514	0.010	1.04	0.33, 3.26
	≥ 15	7	749	0.009	--	
Parasite count	<5000	9	532	0.017	--	
(/ μL)	5000 - 50000	7	737	0.009	0.57	0.21, 1.51
	≥ 50000	1	108	0.009	0.55	0.07, 4.31
[#]Antifolate	Wild	0	26	0.000		
mutations	1-2	16	267	0.057		
	3-4	0	17	0.000		
Fever	Yes	14	955	0.014	2.05	0.59, 7.09
(≥37.5°C)	No	3	422	0.007	--	
Previous	Yes / unknown	0	23	0.000		
drug intake	No	17	1329	0.013		
Artesunate	≥ 3.5 mg	11	1130	0.010	--	
underdosing	3-3.5mg	3	112	0.026	2.71	0.77, 9.56
	<3mg	2	23	0.080	8.30	1.94, 35.5

*per protocol
[#]dhfr+dhps

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TABLE B.3. Multivariate predictors of *P. falciparum* PCR-corrected treatment failure among patients in studies of the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

Predictor	Value	Therapeutic efficacy	
		RR	95%CI
Artesunate dose	≥3.5	--	
(mg/kg)	3-3.5	2.45	0.58, 10.3
	<3.0	6.10	1.04, 35.8
Age	<5	4.46	1.35, 14.7
(years)	≥5	--	
Parasite count	≥5000	0.47	0.22, 1.00
(/µL)	<5000	--	
Febrile	Yes	3.94	1.40, 11.1
	No	--	

TABLE B.1S. Site-wise results of therapeutic efficacy and parasite clearance in studies of the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

State/UT	District/city	Drug	n	Therapeutic Efficacy										PCT (hrs)			
				ACPR	ETF	LCF	LPF	LFU	WTH	Pv	Pf*	Surv	95%CI	≤24	24-48	48-72	≥72
Andhra Pradesh	Vishakhapatnam	ASP	55	52	0	0	0	3	0	0	0	100	--	46	8	1	0
Assam	Nagaon	ASP	51	51	0	0	0	0	0	0	0	100	--	49	2	0	0
Chhattisgarh	Bilaspur	ASP	70	67	0	0	0	2	1	0	0	100	--	64	5	0	0
	Kanker	ASP	69	66	0	0	0	3	0	0	0	100	--	57	11	1	0
DN Haveli	Silvasa	ASP	86	78	0	0	1	1	5	1	0	98.8	91.5, 99.8	19	31	29	1
Goa	North Goa	CQ	55	52	0	0	0	2	1	0	0	100	--	7	33	13	0
Gujarat	Surat	ASP	82	77	0	3	0	2	0	0	0	96.3	89.0, 98.8	25	35	22	0
Jharkhand	Dumka	ASP	56	56	0	0	0	0	0	0	0	100	--	1	29	26	0
	Latehar	ASP	72	70	0	0	0	2	0	0	0	100	--	42	29	0	0
	Ranchi	ASP	71	69	0	0	0	1	0	0	1	100	--	68	3	0	0
	Simdega	ASP	42	39	0	0	0	2	1	0	0	100	--	1	9	32	0
Karnataka	Gulburga	CQ	80	69	0	0	0	10	1	0	0	100	--	3	25	44	3
	Mangalore	CQ	75	64	0	0	0	0	11	0	0	100	--	10	30	21	3
Madhya Pradesh	Betul	ASP	75	68	2	0	0	5	0	0	0	97.3	89.6, 99.3	6	42	22	4
	Chhindwara	ASP	40	39	0	0	1	0	0	0	0	97.5	83.6, 99.6	18	15	7	0
Maharashtra	Gadcharoli	ASP	82	75	0	1	4	1	1	0	0	93.8	85.6, 97.4	53	25	2	0
	Mumbai	ASP	87	86	0	0	1	0	0	0	0	98.9	92.1, 99.8	55	31	1	0
Meghalaya	West Garo Hills	ASP	25	25	0	0	0	0	0	0	0	100	--	21	4	0	0
Orissa	Angul	ASP	65	56	1	0	0	4	0	3	1	98.4	88.9, 99.8	55	5	0	1
	Dhenkanal	ASP	64	56	0	0	1	3	1	3	0	98.3	88.8, 99.8	41	16	3	0
	Sambalpur	ASP	68	65	0	0	0	0	3	0	0	100	--	35	29	1	0
Rajasthan	Baran	ASP	67	60	0	0	0	1	6	0	0	100	--	46	16	0	0
	Pratapgarh	ASP	69	60	0	0	0	0	9	0	0	100	--	46	13	1	0
West Bengal	Jalpaiguri	ASP	80	75	0	0	1	3	0	0	**1	98.7	91.3, 99.8	65	12	3	0
	Kolkata	ASP	78	77	0	1	0	0	0	0	0	98.7	91.3, 99.8	60	14	3	0

*PF-reinfection, **untypeable

TABLE B.3S. Molecular markers of anti-folate resistance in *P. falciparum* isolates collected through the National Antimalarial Drug Resistance Monitoring System, India, 2009-2010

Result	dhfr		dhps	
	n	Haplotype	n	Haplotype
Quadruple	1	**S108N**/**C59R**/**N51I**/**I164L**		
Triple	6	**S108N**/**C59R**/**N51I**/I164L		
Triple	4	**S108N**/**C59R**/N51I/**I164L**		
Double	235	**S108N**/**C59R**/N51I/I164L		
Double	1	**S108N**/C59R/N51I/**I164L**	1	S436F/**A437G**/**K540E**
Single	61	**S108N**/C59R/N51I/I164L	4	S436F/**A437G**/K540E
Single	7	S108N/**C59R**/N51I/I164L	1	S436F/A437G/**K540E**
Wild	27	S108N/C59R/N51I/I164L	255	S436F/A437G/K540E

*Mutations in bold

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APPENDIX C: REVIEW OF ANTIMALARIAL DRUG RESISTANCE IN INDIA

Antimalarial Drug Resistance Of *Plasmodium falciparum* In India 1978-
2007: Changes Over Time And Space

by

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Abstract

Background

After the launch of the National Malaria Control Programme in 1953 shortly after independence, reported malaria cases in India fell to an all-time low of 0.1 million in 1965. However, the initial success could not be maintained and a resurgence of malaria began in the late 1960s. Chloroquine resistance in *Plasmodium falciparum* was first reported in 1973 and increases in antimalarial resistance, along with rapid urbanization and labour migration, complicated the challenge that India's large geographic area and population size already pose for malaria control. Although several institutions have conducted drug resistance monitoring in India, a complete analysis of countrywide data across institutions has been lacking.

Objective

We undertook a systematic review of falciparum malaria drug efficacy studies in India to summarize drug resistance data and describe changes over the past thirty years in order to inform future policy.

Methods

We searched Medline and retrieved unpublished studies from the antimalarial drug resistance unit of the National Vector Borne Disease Control Programme for chloroquine and

sulfadoxine-pyrimethamine (SP) efficacy data from 1978–2007. Only studies with at least 30 patients completing a minimum follow-up of seven days and treated with the correct dose over a standard duration (25mg/kg over three days and 25/1.25mg/kg stat respectively) were included. Treatment failure by drug was compared by region, year, and follow-up length of each study.

Results

We identified 363 *in vivo* efficacy studies involving 18,620 patients. The median proportion of chloroquine failure in a 28-day follow-up was 35% (25–75th percentile: 13–58) and the proportion of studies with chloroquine failures exceeding 10% rose from 12% (2/17) in 1978–79 to 88% (35/40) in 2006–07 (p<0.0001). The median proportion of SP failure was 15% (25–75th percentile: 1–33) and SP resistance increased from 8% in 1984–96 to 26% in 1997–07 (p<0.0001), largely in the Northeast. The extent and rate of chloroquine resistance varied by region and, along with resistance to SP, was greatest in Arunachal Pradesh near the Burmese border.

Conclusions

The results indicate the continued use of chloroquine for the treatment of *P. falciparum* malaria in India would be ineffective. Resistance to SP should be closely followed to protect the efficacy of artesunate+SP, which is the new first-line treatment for falciparum malaria. Strategies to reduce the emergence and spread of future drug resistance need to be pro-active and supported by intensive monitoring.

Introduction

The Republic of India represents the largest population at risk for malaria in the world with 85% of the populace residing in malarious zones (85). Both major *Plasmodium* species,

six primary malaria vectors, several ecotypes including urban malaria, and transmission

intensities ranging from unstable to hyper endemic highlight the challenging malaria

epidemiology in the nation. At the time of independence in 1947, the Indian people were

suffering from an estimated 75 million cases and 800,000 deaths per year (87). After

independence health care was prioritized and the control of malaria was one of their key

aims. In 1953, the National Malaria Control Programme was launched and protected a

population of approximately 165 million with DDT spraying (82,161).The control

programme evolved into the National Malaria Eradication Programme in 1958. Reliable

surveillance gradually developed during the eradication period and the programme seemed to

be highly effective with only 99,667 malaria cases and zero deaths reported in 1965.

However, the long-term success of malaria control could not be sustained. Increasing

insecticide resistance in mosquitoes, urbanization, development projects, population

migration, integration with the general health services, financial difficulties and other

operational challenges laid the foundation for a resurgence of malaria. In 1976, malaria cases

reached a post-eradication peak of 6.47 million cases (88). A new strategy, the Modified Plan

of Operation, was introduced in 1977 after which there has been a steady decline in malaria

cases in the country with only 1.4 million reported cases in 2007. However, the decline in

malaria was not equal in both *P. vivax* and *P. falciparum* species. Around this period, the

proportion of *P. falciparum* cases began to increase and comprised 49% of the total burden in

2007, up from 13% in 1978 even while the rate decreased from 0.90 to 0.67 cases per 1000

during the same interval (figure 1 – National Vector Borne Disease Control Programme

data). The changing species dynamics is a cause for concern as *P. falciparum* is associated

with higher mortality and rapid development of resistance to new drugs. Increasing drug

resistance in *P. falciparum* is thought to be among the causes for the changing scenario in India (162).

Sehgal et al. first documented chloroquine resistant *P. falciparum* in the Northeast in Karbi-Anglong district of Assam in 1973 (163). Routine monitoring of antimalarial resistance using *in vivo* efficacy trials was initiated in 1978 using 13 regionally-based drug resistance teams. While several drug resistance monitoring protocols have been used in the past three decades, the test system generally involves the inclusion of patients with defined criteria, supervised treatment and follow up for clinical and parasitological outcomes. The first reports of sulfa-pyrimethamine (SP) resistance emerged, again from Karbi-Anglong in Assam in 1979 (164). A national antimalarial policy was introduced in 1982 to improve malaria case management and established SP as the treatment for chloroquine resistant areas. Drug efficacy monitoring by the national programme and others has provided data to guide treatment strategy and update policy. Artesunate+sulfadoxine-pyrimethamine replaced SP as the second-line drug in 2005 for use in treatment failures and as the primary antimalarial in areas with documented drug resistance. In 2007, artesunate+SP was selected to be deployed as the first-line therapy in high risk districts in addition to identified resistant areas, with the goal of covering most of the nation's *P. falciparum* burden, and in 2010 became the universal first-line therapy.

The public health system responds to antimalarial failures with evidence-based policy; however, variations in resistance within the country and a diverse malaria situation complicate decision-making. Furthermore, drug resistance studies have been conducted by various institutions and a complete analysis of Indian data across institutions is lacking. A systematic review was undertaken to (i) summarize antimalarial drug resistance data; (ii)

describe temporal and spatial trends which can inform future policy; and (iii) identify gaps in understanding.

Methods

Search and selection

We focused our search on the drugs chloroquine and SP and on *P. falciparum*. Few efficacy trials exist for other antimalarial compounds in India and none in regards to routine monitoring. Resistance to mefloquine and quinine is reported but appears to be rare (165) and the cases are not well documented. Trials using artemisinin combination therapies in India have consistently demonstrated treatment success above 95% (166,167).With regards to vivax malaria, only a few case reports of chloroquine resistance (namely Mumbai, Uttar Pradesh, and Bihar) exist. Contrary to these reports, systematic trials from across the country have reported 100% efficacy of the standard dose of chloroquine (25mg/kg over three days) (168). At present, chloroquine resistant *P. vivax* is not a major concern for India.

We reviewed data collected between 1978 and 2007 (the year drug policy changed) from published and unpublished sources. First, we searched the PubMed/Medline database through June 1, 2008 with the following terms: ("India") AND ("malaria" OR "falciparum" OR "Plasmodium falciparum") AND ("resistance" OR "resistant" OR "failure" OR "efficacy" OR "sensitivity"). There were no language restrictions to the search. Second, we retrieved unpublished data from the National Vector-Borne Disease Control Programme, National Institute of Malaria Research, and the World Health Organization headquarters as well as its Southeast Asia Regional Office. We examined the abstracts (or titles if the abstracts were unavailable) to identify published articles that mentioned any type of *P. falciparum* resistance (in vivo, in vitro, molecular) to chloroquine or SP. Next, we undertook

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a detailed manual review and excluded articles or unpublished studies that (1) did not describe a minimum of seven days of follow-up for chloroquine and 28 days for SP (2) did not use standard dosing, i.e. 25mg/kg over three days for chloroquine, or 25/1.25mg/kg as a single dose for sulfa-pyrimethamine (3) had less than 30 patients complete follow-up (4) included patients with severe malaria (5) included recrudescent patients (6) did not provide exact or graphical data regarding the number of patients who completed follow-up and number of treatment failures (7) could not geographically disaggregate results to at least the district level.

Data extraction

Two investigators searched for and recorded the total number of studies. One investigator merged the datasets and extracted the following information for each study: study year, administrative divisions (state, district), number of patients, total number of failures, duration of follow-up, and PCR correction if any. For studies spanning more than one year, the start year of the study was recorded. The smallest unit of geography recorded was the district (second level administrative unit after the state level, with approximately 1 to 3.5 million population). In terms of healthcare provision the district is the smallest unit at which at least a public secondary care facility is available. Studies spanning multiple areas of the same district but in the same year and by the same investigator were aggregated. Each study was assigned a geographic region based on states: East (Andaman & Nicobar Islands, Bihar, Chhattisgarh, Jharkhand, Orissa, West Bengal), North-central (Haryana, Madhya Pradesh, Uttar Pradesh), Northeast (Arunachal Pradesh, Assam, Meghalaya, Mizoram, Nagaland, Tripura), South (Andhra Pradesh, Karnataka, Tamil Nadu) and West (D & N Haveli, Goa, Gujarat, Maharashtra, Rajasthan).

Drug resistance monitoring

Since 2005 the policy on the cut off needed for the routine use of second-line malaria treatment in an area has been more than 10% total treatment failure in that locale in a sample of minimum 50 patients.[14] Before adopting this threshold, routine drug efficacy monitoring was conducted according to *in vivo* protocols of the World Health Organization. However, most 7-day trials were conducted according to the method of Prasad et al. (169) and the cut off point for treatment change was 25% RII+RIII resistance in a minimum of 30 patients, which is comparable to early failures according to new protocols. Currently, the WHO standard therapeutic efficacy test provides the information for deciding the change of treatment policy. The duration of follow up is determined by half-life of the drug being tested but was 28 days for chloroquine and SP studies conducted through routine monitoring. The classification of therapeutic response varied from study to study with most using the prevailing WHO criteria of their time. Presently, outcomes are classified as early and late treatment failures or adequate response according to the most recent guidelines (117). For our purposes we used a composite definition of treatment failure which included the classical RI, II, III criteria and the revised WHO criteria of 1996 which forms the basis of current standards. For 28 day follow-up studies this covers recrudescence after clearance of parasitemia (RI, late treatment failure), reduction in parasitemia by >75% of baseline without clearance (RII, early treatment failure), failure to reduce parasitemia to <25% of baseline (RIII, early treatment failure), and fever or signs of severe malaria. The follow-up day at which each criteria was assessed varied between the classical, which collected daily blood smears up to day 7, versus the revised protocol, which does not. We used the outcome designated at the time of the study since detailed data on individual patients was not available precluding the possibility of reclassification.

Analysis

The proportion of treatment failures was calculated per protocol for each study. The total number of treatment failures (uncorrected by PCR) was divided by the number of patients excluding those lost to follow-up. We analysed the proportion of treatment failures by study state, region, decade, and follow-up period. We examined the change in chloroquine resistance by plotting the proportion of failures in each 28 day study by the study year. Since the present strategy is to change policy at more than 10% level, the comparison of the proportion of 28 day studies exceeding a certain proportion of failures by year was done using this cut off. We used Fisher's exact test to analyse the change in the proportion of studies exceeding the 10% treatment failure cut-off from when routine monitoring began to the end of the study period. The change in the proportion of SP failures was calculated using the chi-square test. To determine differences in chloroquine treatment failure proportions between regions we performed a Kruskal-Wallis nonparametric test for K independent samples using SPSS version 14.0. The study periods for comparison were determined by observing the slope of change in the proportion of treatment failures. To produce a national map of existing resistance, we identified districts where a study exceeded 10% treatment failure in any length of follow-up. If a high proportion of failures occur in a short study, even more failures would be expected in a longer follow-up period. Districts endemic for *P. falciparum* were identified by examining surveillance data and selecting areas reporting an annual incidence >1 per 100,000 population. Maps were produced using Health Mapper software (Version 4.2, World Health Organization, Geneva).

Results

The literature search of the PubMed/Medline database produced 738 articles of which 649 were not eligible for detailed review (figure 2). The abstracts of ineligible articles addressed various topics including *P. vivax* malaria, experimental compounds, insecticide resistance, etc and therefore did not pertain to the topic of interest. Of the 89 articles manually reviewed, 41 were included in the review. Some articles reported the results of multiple studies (i.e. *in vivo* trials conducted in different districts). We also collected 334 unpublished studies of which 315 were included in the analysis. Among both published and unpublished sources, not all studies reported the number lost to follow-up. Some studies included single-dose primaquine (0.75 mg/kg) for gametocytocidal effect as per national policy. PCR-corrected results were available for three studies (but were not used in our analysis). In these studies, the proportion of treatment failures classified as reinfections ranged from 0 to 8%, but not all samples were successfully genotyped. As malaria transmission in most of India is low, we expect low rates of reinfection. More studies have been undertaken recently with 161 trials since 2000 compared to 121 in the 1990s, 64 in the 1980s, and 18 in the late 1970s. 134 studies were conducted in the eastern region, 72 in the northeast, 64 in the west, 60 in the south, and 34 in the northcentral area. There were 119, 4, and 240 studies with 7, 14, and 28 days of follow-up respectively.

Chloroquine resistance

337 studies of chloroquine efficacy with 17,189 patients were recorded. The number of studies and proportion of failures varied between regions and in states within a region (table 1). The median proportion of chloroquine failure was 35.1% (25–75th percentile: 13.0–58.2) in studies with a 28 day follow-up period. Studies with 7-day follow up, which largely

detected early treatment failures, were phased out beginning in 2000 and the last seven day study was conducted in 2003. The proportion of failures detected is higher in 28 day follow-up studies than in 7 day follow-up studies conducted in the same areas since late treatment failures are detected in the former.

Studies conducted between 1978 and 2007 show a trend of an increasing proportion of failures to chloroquine over time (slope=0.73, r^2=0.07) (figure 3). When routine monitoring of drug resistance began in 1978-79, 2 out of 17 studies, 12%, exceeded the 10% threshold currently used in India to switch an area to the second-line treatment. In 2006-07, the proportion of chloroquine studies exceeding 10% treatment failure increased to 88% (35 out of 40 studies, p<0.0001) (figure 4).

Regional variation in chloroquine resistance

Drug efficacy studies of chloroquine, with a minimum of 30 patients in any period of follow-up, have exceeded 10% treatment failure in 115 districts (figure 5). These districts represent 20 out of 28 states and 2 of 5 union territories. The remaining states and territories either have little *P. falciparum* and/or have not undertaken any trial of antimalarial drug resistance. There were eight districts in which all drug efficacy studies conducted did not have more than 10% treatment failure. However, only three of the studies were conducted since 2000 and there may be subsequent changes in parasite drug sensitivity since the study was performed. From 1978-1985, the median proportion of treatment failure between regions (Northeast > East > West > South > Northcentral) were significantly different (χ^2=20.45, p=0.001). For the data from 1986–2007, there is a similar trend but the difference was not statistically significant (χ^2=8.91, p=0.06) as the median proportion of treatment failures increased substantially in all regions.

Sulfa-pyrimethamine resistance

Between 1978 and 2007, 26 studies of SP efficacy with 1431 patients of SP were conducted. Three studies, all in Assam, used sulfalene (Karbi-Anglong 1984, Nalbari 1992, Darrang 1993), while the rest used sulfadoxine. The median proportion of SP failure was 15.0% (25–75th percentile: 0.7–33.1) in 28 days of follow-up. SP resistance increased from 7.7% (19/246) in 1984–96 to 25.9% (307/1185) in 1997-07 (χ^2=38.29, p<0.0001) (table 2). Most of the studies were conducted in northeastern states and Arunachal Pradesh, which is along the Chinese and Burmese borders, displayed the highest SP failure rate.

Discussion

Efficacy studies on antimalarial drugs conducted in India since 1978 indicate that *P. falciparum* resistance to chloroquine increased over time and is present across all regions of the country. The efficacy of sulfa-pyrimethamine for *P. falciparum* was reduced in some recent studies, largely in the Northeast.

Chloroquine resistance: changes over time

Nationally, treatment failures of chloroquine increased between 1978 and 2007. However, the drug resistance monitoring programme was designed to aid in the detection of "foci" of antimalarial drug resistance for the change of treatment policy. Longitudinal trends were only obtained in a few areas. Study sites were purposively sampled using a number of criteria: clinical case reports, outbreak prone areas, high *P. falciparum* burden, development project areas, reported malaria fatalities, etc. Thus, in each year there was broad variation in treatment failure as studies were conducted in diverse settings across the sub-continent. The

heterogeneity in the location of and frequency of studies created challenges in aggregating data and drawing broader conclusions. Yet, we observed an increase in the proportion of failures over time. This trend persisted with the inclusion of 7-day follow-up studies, in which we found the mean proportion of failures was lower due to a shorter follow-up where late treatment failures were not detected. The proportion of studies exceeding a pre-defined threshold of treatment failures also increased over time. Before 2005, 25% treatment failures in a study dictated the need to change the first-line malaria treatment for an area. A 10% threshold was adopted in line with WHO recommendations and it was demonstrated as a cost-effective cut off in India (170). In 2006–07, 88% of trials conducted exceeded the 10% treatment failure threshold necessary for switching an entire area to the second-line drug. This suggests further studies of chloroquine resistance, in the few areas remaining to be phased into artesunate+SP treatment, will have limited utility as nearly all trials detected more than 10% treatment failure. Finally, the increase in chloroquine-resistant *P. falciparum* has contributed to the growing proportion of *P. falciparum* within total malaria cases (171). Changes in the proportion of *P. falciparum* could serve as an indicator of the effects of the broader use of artesunate+SP which is now deployed in areas covering more than 90% of the reported national *P. falciparum* burden.

Chloroquine resistance: changes over space

The median proportion of chloroquine resistance initially varied by region. Most treatment failures occurred in the Northeast where the original focus of drug resistance was found, followed by the eastern and then western regions. Drug resistance in the northeast area likely originated from neighbouring countries (Thailand 1962, Myanmar 1969, Bangladesh 1970) which reported chloroquine failures before India (172,173). Resistant parasite strains

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may have then spread across India through host movements, particularly the migrant labour which travels from eastern India to the western states (174). Thus, chloroquine resistance in India is now widely distributed and our data indicates a geographic clustering of resistant areas rather than isolated foci. Most places in the nation which have not been identified as chloroquine resistant have not had a drug efficacy study conducted there. Changing treatment policy to a second-line drug at the level of the primary health centre, block, or cluster of blocks has helped India limit malaria mortality and morbidity among those patients who receive care at government facilities. However, it does not appear to have stemmed the spread of antimalarial drug resistance. In the face of continued selection pressure, drug resistance strains will spread. Without major geographic barriers, transportation between nearby areas and interactions between catchment populations assures an exchange of vectors and parasite strains. Even when drug sensitivity remains, if the area is in proximity to sites with drug resistance then strains will intermingle and in the face of continued chloroquine use resistant strains will proliferate due to a competitive advantage. On the other hand, effective treatments implemented on larger scales can lower malaria transmission and may even reduce existing drug resistance as described in other settings (175–177). Thus, minimizing uneven drug pressure by deploying an effective drug at a larger geographic unit such as the district, or even cluster of districts, is a better strategy to prevent the spread of drug resistance. A larger area for treatment policy change will also necessitate fewer efficacy studies and reduces operational challenges related to drug supply.

Sulfa-pyrimethamine resistance

Similar to other control programmes, the dose and choice of sulfa derivative used in India evolved over time. Originally, several sulfa derivatives were deployed including

sulfalene but these were gradually replaced by sulfadoxine. The two tablet adult dose was found to be as efficacious as the three tablet dose in initial trials; however, the latter became the standard after some period. The studies we surveyed were conducted with the full dose only. Sulfa-pyrimethamine efficacy was reduced in parts of the country, particularly the Northeast region. These study results may be a conservative estimate as SP failures can occur after 28 days, and a 42-day follow-up is now recommended where possible. Since a more widespread use of SP would increase resistance, artesunate+SP replaced the monotherapy as combination treatments, particularly artemisinin-based therapies, are touted as a means for both the provision of effective treatment and the prevention of drug resistance (135). There is a concern that as the partner drug, pre-existing resistance to SP could compromise the combination therapy. Additionally, while age group blister packs of artesunate+SP are being made available, in principle, a co-formulated drug would be ideal. Although these constraints were recognized at the time of the inclusion of artesunate+SP into the national drug policy, this was the only option available as co-formulated ACTs were not registered. Treatment with artesunate+SP has demonstrated adequate safety and efficacy till date in India and is available in sufficient quantities. Recently, trials with several fixed-dose combinations such as artesunate-mefloquine, artesunate-amodiaquine, artemether-lumefantrine, dihydromisinin-piperaquine and artesunate-pyronaridine have been completed to provide local efficacy data for other options. Determining and preparing "back-up" combination therapies is high priority. In the meantime artesunate+SP efficacy requires careful monitoring.

Limitations of the study

Our study has two main limitations. First, a range of quality control issues varied between studies or were not reported. These include selection criteria, microscopy technique,

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drug quality, record keeping, etc. Our results are derived from a per protocol analysis and differences in the amount of and reasons for loss to follow-up could impact estimates of treatment failure. While exact numbers are not available for most of the studies, in our experience loss to follow-up rarely exceeded 10% and in most studies was zero. This is due to the mass fever survey approach used for recruitment in many studies. The study team enrolls the entire study population at once and then remains in the area until the completion of the trial. Second, the variability in the study methodologies is relatively minor compared to differences in the number of studies and their location each year. The diverse range of years and sites in which studies were conducted make data aggregation difficult. Previously discussed operational factors dictated the number and location of studies. Treatment failure is an indirect measure of true parasite resistance, as host immunity and pharmacokinetics also determine outcomes. Expected variation in the latter factors between different areas of the country could be responsible for some of the variation in treatment failure rather than any difference in resistance. Caution should be exercised in attempting to interpret any precise summary estimate of treatment failure. Thus, we did not produce a meta-analysis but rather focused on overall trends through a systematic review. The large number of studies analysed should mitigate the limitation posed by the temporal and spatial variability of the data.

Current Indian initiatives against drug resistance

The National Vector Borne Disease Control Programme has responded to the challenge of drug resistance with several strategies. Rational drug use is being promoted in order to reduce the overall drug pressure. This involved the phasing out of presumptive therapy, strengthening of microscopy services, and extending the availability of rapid diagnostic tests to peripheral areas using the nationwide community health worker (ASHA)

system under the National Rural Health Mission (178). Second, inadequate dosing or the use of improper therapies, such as the use of parenteral artemisinin derivatives for treating uncomplicated malaria, can promote resistance. New guidelines have been developed in cooperation with the National Institute of Malaria Research which present the treatment policy in an accessible format for public and private physicians in order to improve compliance. Regulation banning the sale of oral artemisinin monotherapy is in place. A group of 15 alternating sentinel sites were selected in 2009 to enable the longitudinal monitoring of antimalarial drug resistance. Molecular techniques such as PCR correction of treatment failures are now used in antimalarial drug efficacy trials with help of research institutions such as the National Institute of Malaria Research.

Future steps

Further research and programme efforts are needed to combat antimalarial drug resistance in India. Previously, with chloroquine treatment the inclusion of primaquine as a gametocytocidal agent was standard. Artemisinin combination therapies exhibit useful gametocytocidal properties but do not eliminate mature gametocytes. It is not clear whether the addition of single-dose primaquine to artesunate+SP will impact malaria transmission or the spread of drug resistant strains in India. *In vitro* susceptibility and molecular markers reflect intrinsic resistance to a drug and their changes precede clinical resistance in the field (179). Molecular and *in vitro* monitoring could supplement efficacy trials and provide early warning regarding emerging drug resistance. Finally, the introduction of resistant malaria into non-immune populations such as refugees or migrants increases the opportunity for manifestation and spread of resistance, because parasites with low or moderate resistance would be cleared in semi-immune populations (180). In India, antimalarial resistance for

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chloroquine and SP was first reported near and exhibits an increasing gradient towards the international border with Myanmar (181). Now reports of artemisinin "tolerance" along the Thai-Cambodia border and the historical westward spread of drug-resistant strains generate concern about the long-term efficacy of artemisinin combination therapies in India (147). Best practices for malaria control along border areas and in migrant populations in India need to be determined. Overall, a robust and specific plan to combat drug resistant parasites will be fundamental in fulfilling our commitment to control malaria in India.

FIGURE C.1. The incidence of and proportion of *P. falciparum* malaria cases in India between 1961-2007. Source: NVBDCP
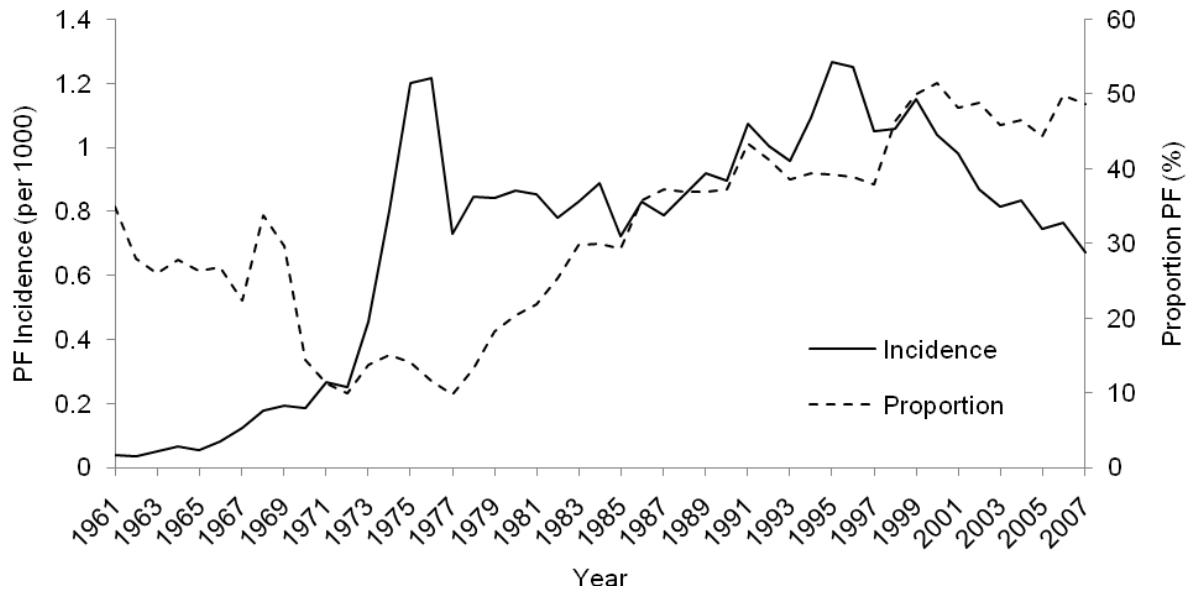
FIGURE C.2. Search strategy and selection criteria

738 potentially relevant articles retrevied from PubMed database

649 articles were excluded after reviewing the abstract (or title if abstract was unavailable)

89 articles and 334 unpublished studies eligible for detailed review

48 articles and 19 unpublished studies did not meet inclusion criteria

41 articles and 315 unpublished studies included in the systematic review

FIGURE C.3. Chloroquine resistance studies with 28-day follow-up by the proportion of treatment failures in India between 1978 and 2007
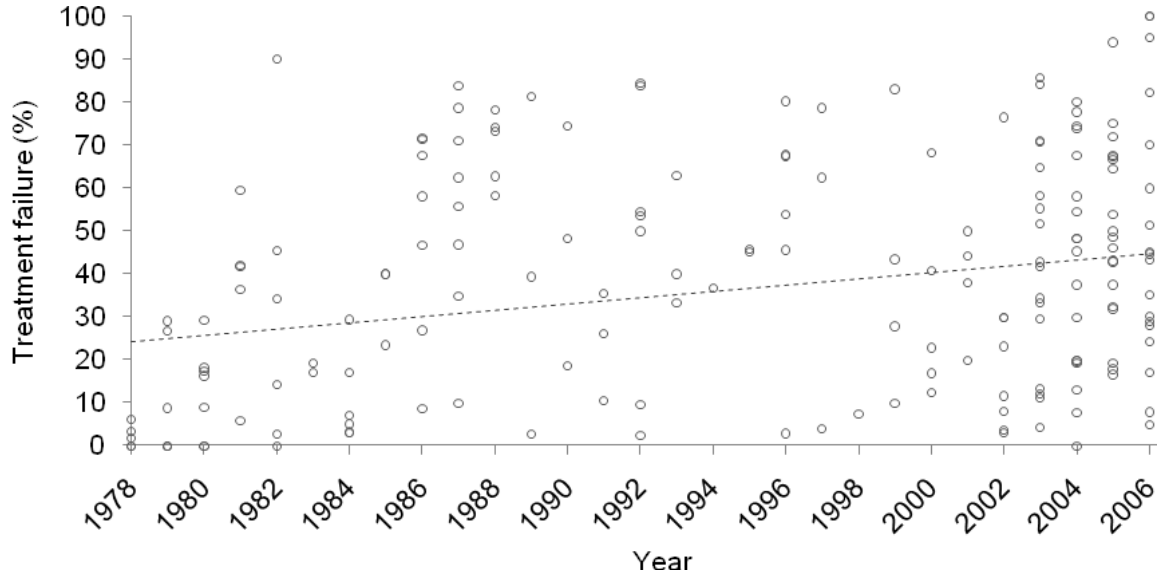
FIGURE C.4. Number of 28-day chloroquine resistance studies in India between 1978 and 2007 by the proportion of treatment failures exceeding 10%

FIGURE C.5. – Districts in India with chloroquine treatment failure >10% (red) in any study between 1978 and 2007 and present *P. falciparum* endemic areas (gray lines)

TABLE C.1. Results of chloroquine efficacy studies conducted in India between 1978 and 2007 by area, year and duration of follow-up

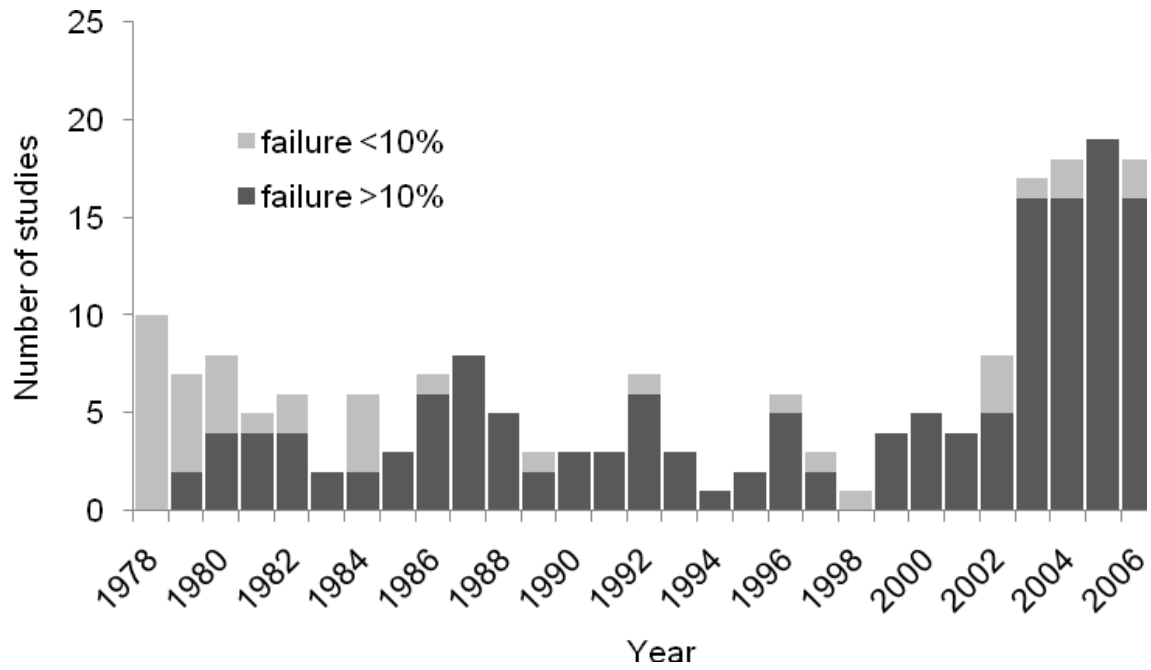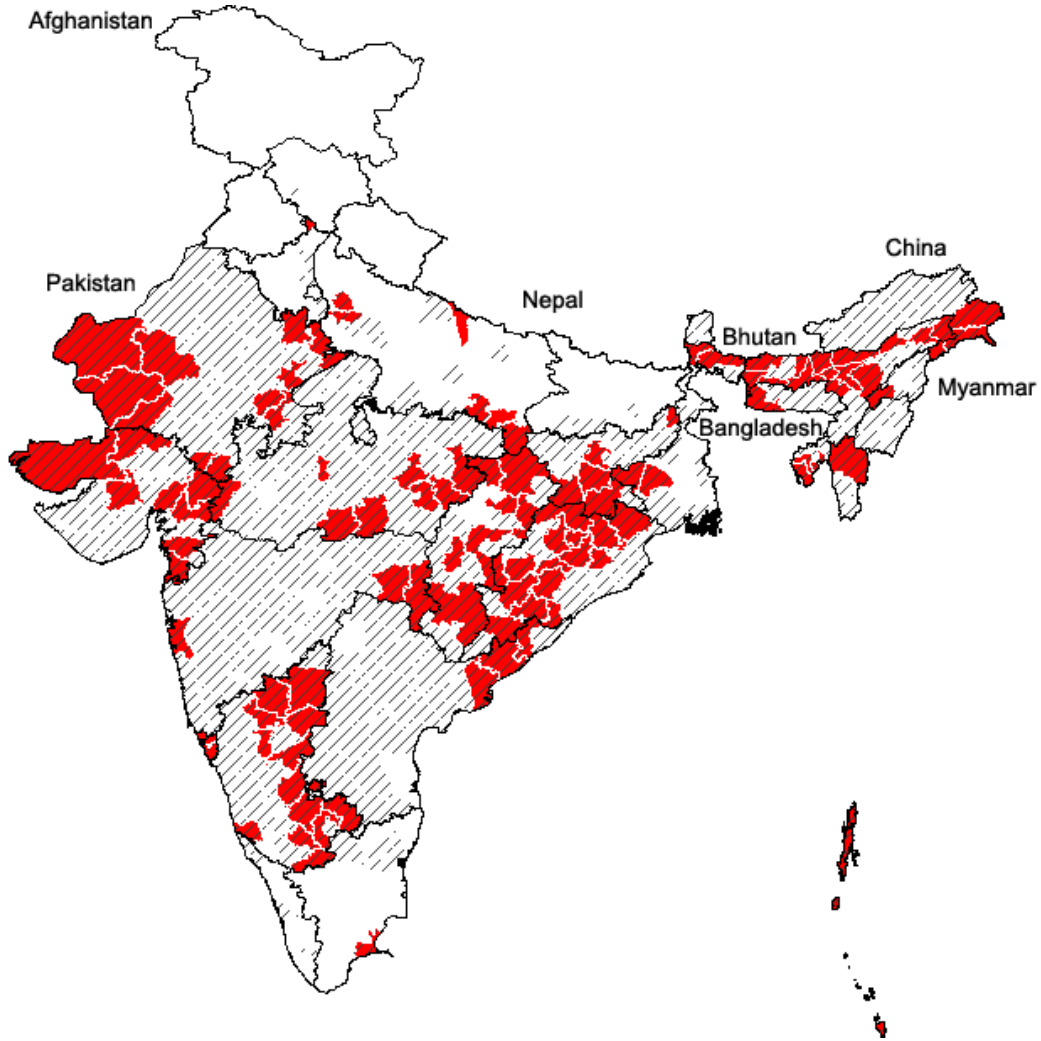|	all follow-up*				7d only				28d only			
	studies	n	failures	%	studies	n	failures	%	studies	n	failures	%
Region (state)												
Northcentral	33	2723	687	25	16	741	116	16	17	1982	571	29
Haryana	3	113	21	19	1	38	0	0	2	75	21	28
Madhya Pradesh	17	1922	556	29	12	591	86	15	5	1331	470	35
Uttar Pradesh	13	688	110	16	3	112	30	27	10	576	80	14
Northeast	54	2541	893	35	13	446	58	13	38	1946	759	39
Arunachal Pradesh	7	290	174	60	1	30	1	3	6	260	173	67
Assam	37	1909	607	32	10	356	54	15	24	1404	477	34
Meghalaya	5	159	20	13	2	60	3	5	3	99	17	17
Mizoram	1	47	28	60					1	47	28	60
Nagaland	1	34	10	29					1	34	10	29
Tripura	3	102	54	53					3	102	54	53
East	126	5830	1471	25	61	2613	299	11	64	3079	1130	37
A&N	2	115	15	13					2	115	15	13
Bihar	1	37	0	0	1	37	0	0				
Chhatisgarh	12	454	87	19	4	141	9	6	8	313	78	25
Jharkhand	21	971	212	22	13	588	67	11	8	383	145	38
Orissa	58	2914	719	25	23	1113	122	11	34	1663	555	33
West Bengal	32	1339	438	33	20	734	101	14	12	605	337	56
South	60	3124	1007	32	6	277	20	7	54	2847	987	35
Andhra Pradesh	17	775	95	12	5	236	20	8	12	539	75	14
Karnataka	39	2184	836	38	1	41	0	0	38	2143	836	39
Tamil Nadu	4	165	76	46					4	165	76	46
West	64	2971	1074	36	23	1049	198	19	41	1922	876	46
DN Haveli	3	175	19	11	2	121	12	10	1	54	7	13
Goa	4	183	96	52	1	34	10	29	3	149	86	58
Gujarat	29	1297	392	30	13	645	141	22	16	652	251	38
Maharashtra	9	594	297	50	1	45	2	4	8	549	295	54
Rajasthan	19	722	270	37	6	204	33	16	13	518	237	46
Year												
1978-9	18	783	59	8	1	39	0	0	17	744	59	8
1980s	63	3900	1292	33	10	466	65	14	53	3434	1227	36
1990s	114	5265	1190	23	81	3578	475	13	33	1687	715	42
2000-7	142	7241	2591	36	27	1043	151	14	111	5911	2322	39
Total	337	17189	5132	30	119	5126	691	13	214	11776	4323	37

TABLE C.2. Results of 28-day SP efficacy studies conducted in India between 1978 and 2007

state (district)	year	n	total failures	percent
Arunachal Pradesh				
Changlang	1992	57	4	7
Changlang	1999	43	19	44
Changlang	2002	65	35	54
Changlang	2006	67	38	57
Lohit	2002	70	12	17
Lohit	2006	212	81	38
Assam				
Darrang	1993	36	0	0
Darrang	2004	37	4	11
Karbi-Anglong	1984	30	8	27
Karbi-Anglong	2001	51	18	35
Nagaon	2002	78	0	0
Nagaon	2003	32	0	0
Nalbari	1992	30	1	3
North Lakhimpur	2007	47	6	13
Sonitpur	2001	49	21	43
Sonitpur	2003	32	0	0
Madhya Pradesh				
Mandla	1997	114	42	37
Orrisa				
Kandhamal	2004	38	1	3
Keonjhar	2002	61	0	0
Sundargarh	1991	60	6	10
West Bengal				
Bankura	2005	35	6	17
Jalpaiguri	1996	33	0	0
Jalpaiguri	2001	58	11	19
Puruilia	2000	30	7	23
Purulia	2003	31	0	0
Total		1431	326	23

APPENDIX D: OTHER PUBLICATIONS

Other Peer-Reviewed Malaria Articles Completed During Doctoral Training, 2009-2012

<u>Published</u>

1. **Shah NK**. Defining and counting malaria deaths. IJMR. 2012 March 135:270-72

2. Mishra N, Anvikar AR, **Shah NK**, Kamal VK, Sharma SK, Srivastava HC, Das MK, Pradhan K, Kumar H, Gupta YK, Gupta P, Dash AP, Valecha N. Prescription practices and availability of artemisinin monotherapy in India: where do we stand? Malar J. 2011 Dec 13;10:360.

3. Chandra S, **Shah NK**, Sriganesh V. Cochrane Students Journal Club - a learning resource for gathering and appraising evidence: An example of rational use of medicines to prevent malaria relapse. Intl Journal of User-Driven Healthcare. 2011 Oct 1(4), 31-41

4. **Shah NK**, Dhariwal AC, Sonal GS, Gunasekar A, Dye C, Cibulskis R. Malaria-attributed death rates in India. Lancet. 2011 Mar 19;377(9770):991

5. Lin JT, Bethell D, Tyner SD, Lon C, **Shah NK**, et al. Plasmodium falciparum gametocyte carriage is associated with subsequent Plasmodium vivax relapse after treatment. PLoS One. 2011 Apr 20;6(4):e18716

6. **Shah NK**. Assessing strategy and equity in the elimination of malaria. PLoS Med. 2010 Aug 3;7(8):e1000312.

<u>Under consideration</u>

1. Anvikar A, Arora U, **Shah NK** et al. Antimalarial Drug Policy in India: Past, Present and Future. *Submitted Indian J of Medical Research*

2. **Shah NK**, Kumar A, Valecha N. India and Global Estimates of Malaria Burden. *Submitted Lancet*

3. Sarkar J, **Shah NK**, Murhekar MV. Incidence of severe malaria and risk factors for death in secondary and tertiary health facilities of Alipurduar, India. *Submitted J Vector Borne Diseases*

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7.   Shah NK. Assessing strategy and equity in the elimination of malaria. PLoS Med. 2010;7(8):e1000312.

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