In Vitro Antimalarial Drug Sensitivity Testing For Plasmodium falciparum and Plasmodium vivax

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Abstract: *Aim of this study was to standardize the Antimalarial drug sensitivity testing using microtitre plate method similar to WHO III plate method. The study was conducted over a period of two years from January 2013 to December 2014. Total 44 patient samples were included in this study (22 were P. falciparum and 22 were P. vivax). 68.18% of P. falciparum blood samples showed resistance to chloroquine. Resistance was also detected for amodiaquine (18.18%) and sulfadoxine / pyrimethamine (15%). No resistance was detected for artemisinin, mefloquine and quinine. P. vivax showed 27.73% resistance to chloroquine and 13.64% resistance to primaquine. No resistance was found for other antimalarial drugs. Chloroquine resistance has developed because of indiscrimate use of this drug. Proper diagnosis of malaria and drug sensitivity testing should be done routinely to prevent emergence of resistance to antimalarial drugs. Monotherapy should be avoided and combined drug therapy will reduce chances of emergence of drug resistance.*
Keywords: *Microtitre plate, Chloroquine, Primaquine, Artesunate, Antimalarial drug sensitivity testing.*

I. Introduction

Malaria still poses a threat to the health of residents and travellers in tropical countries. There are currently six species of the genus Plasmodium known to infect humans: Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale curtisi, Plasmodium ovale wallikeri, Plasmodium malariae and Plasmodium knowlesi. Of these, P.falciparum is the species responsible for most of the mortality and morbidity associated with the disease and it is during the asexual erythrocytic stages that most of the symptoms of malaria are manifest. Studies into many aspects of human malaria parasite biology were greatly enhanced by the development of a method to culture asexual blood stages of P. falciparum in vitro in 1976.[1]

The first in vitro development of malaria parasites was reported nearly 80 years ago by Bass and Johns (1912). They obtained defibrinated blood from patients infected with P. falciparum and cultured the sample at 37°C in a glass vial to which a small amount of glucose had been added. Newly developed rings could be observed after one generation time and only occasionally after one to two additional cycles.[2]

Number of attempts were made to develop a better culture medium including such methods as short term cultures with Harvard growth medium [3,4] and modified Harvard growth medium.[5] However, only when a new medium, RPMI 1640, developed originally for culture of human leukocytes,[6] became available and were proven to be significantly superior to Harvard medium did it allow for a successful continuous culture of the malaria parasite and which is still being widely used throughout the world.[7]

This technique is particularly suitable for small scale cultures, such as those required for drug sensitivity tests, but it is not adequate for large scale cultures where high yield of parasite is needed, for instance, in parasite enzyme isolation and purification and in studies of parasite organelles. Therefore, other techniques were developed for large scale cultures, such as tilting flask [8] and shaken flask [9] methods. Unfortunately, these procedures require wasteful daily changes of medium either by manual or semi-automated methods. A technique for cultivation of P. falciparum without daily medium replacement has been reported which produced high yield of parasites after 3-4 days of cultivation.[10]

Urban malaria is a complicated and severe problem in India and more than 130 towns spread across 17 states are covered by the urban malaria control scheme. The situation has been further complicated by the spread of the strains of the plasmodium species, which are resistant to chloroquine and other antimalarial drugs.[11] The resistance to antimalarial drugs, especially chloroquine (CQ), of Plasmodium falciparum is one of the principal factors contributing to the worldwide increase in morbidity and mortality due to malaria. Different approaches have been developed to monitor the extent of antimalarial drug resistance and to determine the biologic mechanisms by which the parasite has evaded the action of the drug.[12]

II.   Methodology

This prospective study was carried out at Department of Microbiology, MGM Medical College and Hospital, Navi Mumbai, India over a period of two years from January 2013 to December 2014. The control ATCC strain Plasmodium falciparum (3D7) was procured from Haffkine Institute for Training, Research & Testing, Mumbai and Indian Institute of Technology Bombay, Powai, Mumbai, India. Parasitic index was determined before antimalarial drug sensitivity test.

96 wells microtitre plate with lid (sterile) was procured from Genetix Biotech Asia Pvt. Ltd., India. The drugs were diluted as per WHO III plate method (micro test).[26] The diluted drugs (20 µl of each) were loaded in sterile flat bottom microtitre plate and dried to make the drug in powder forms which adhere to the bottom of the plate. One microtitre plate was prepared for each drug. Sufficient stock of microtitre plates were prepared and stored in refrigerator. The drug concentration was used in followings: No drugs in Row A 1 to 12, it contains patient blood and culture medium in ratio of 1:9 after synchronization of malarial parasites.

1. Chloroquine and Primaquine: 20 µl each concentrations of 0.2 (Row B); 0.4 (Row C); 0.8 (Row D); 1.6 (Row E); 3.2 (Row F); 6.4 (Row G) and 12.8 µmol per l (Row H). The concentrations are expressed in µmol / l.
2. Mefloquine: 20 µl each concentrations of 0.4 (Row B); 0.8 (Row C); 1.6 (Row D); 3.2 (Row E); 6.4 (Row F); 12.8 (Row G) and 25.6 µmol per l (Row H). The concentrations are expressed in µmol / l.
3. Quinine: 20 µl each concentrations of 0.08 (Row B); 0.16 (Row C); 0.32 (Row D); 0.64 (Row E); 1.28 (Row F); 2.56 (Row G) and 5.12 µmol per l (Row H). The concentrations are expressed in µmol / l.
4. Amodiaquine: 20 µl each concentrations of 0.05 (Row B); 0.1 (Row C); 0.2 (Row D); 0.4 (Row E); 0.8 (Row F); 1.6 (Row G) and 3.2 µmol per l (Row H). The concentrations are expressed in µmol / l.
5. Artesunate and Arteether: 20 µl each concentrations of 3 (Row B); 10 (Row C); 30 (Row D); 100 (Row E); 300 (Row F); 1000 (Row G) and 3000 nmol per l (Row H). The concentrations are expressed in nmol / l.
6. Sulfadoxine / pyrimethamine: 20 µl each concentrations of 0.2 (Row B); 0.6 (Row C); 2.0 (Row D); 6.0 (Row E); 20.0 (Row F); 60.0 (Row G) and 200.0 µmol per l (Row H). The concentrations are expressed in µmol / l.

Control strains, culture media were added in microtitre plate wells as follows:

Row 1 well A - control strain + culture medium only. Wells B to H - drug dilutions + control stain (20 µl) + culture medium (180 µl) - RPMI-1640 for P. falciparum. Row 2 to 12 contains various patients blood samples + culture medium. The microtitre plate was incubated in CO_2 incubator (adjusted with 5% CO_2) for 48 hours. Red cells settle at bottom. Supernatant media was discarded using micropipette. Smears were prepared from sedimented red cells after 24 hours for P. falciparum and 48 hours for P. vivax. Smears were prepared stained and parasitic index determined and compared with controls and earlier parasitic index.
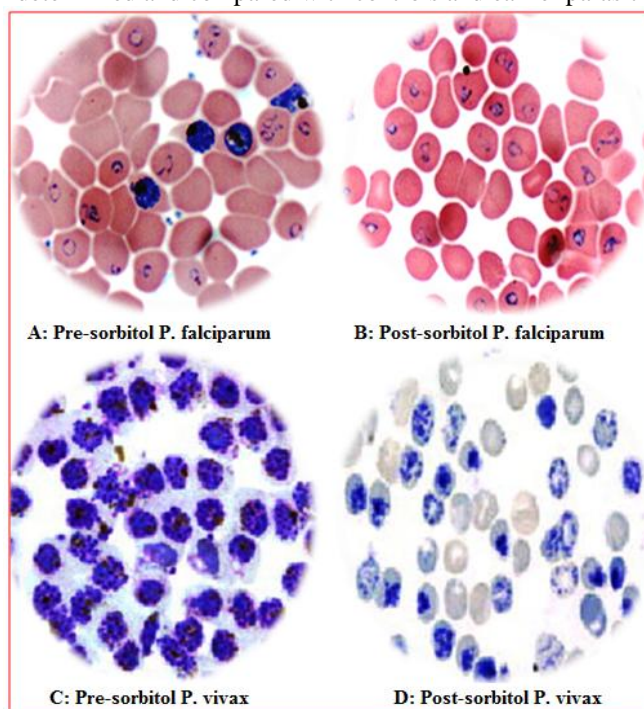

A: Pre-sorbitol P. falciparum   B: Post-sorbitol P. falciparum

C: Pre-sorbitol P. vivax   D: Post-sorbitol P. vivax

Figure 1(A-D): Showing Plasmodium falciparum and Plasmodium vivax before and after treatment with sorbitol.
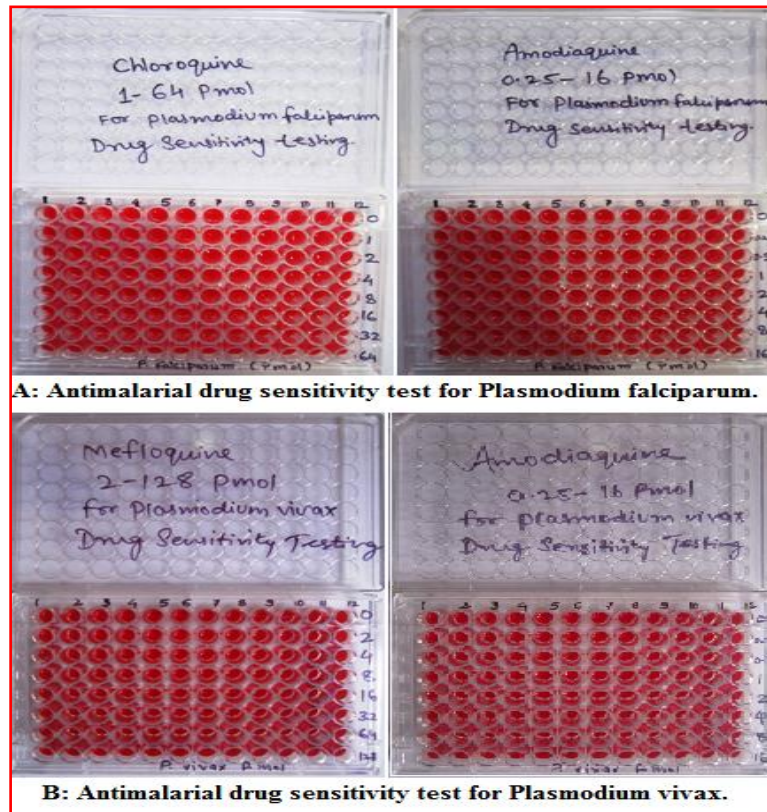
Figure 2(A-B): Showing antimalarial drug sensitivity test for Plasmodium falciparum and Plasmodium vivax similar to micro test WHO III plate method.
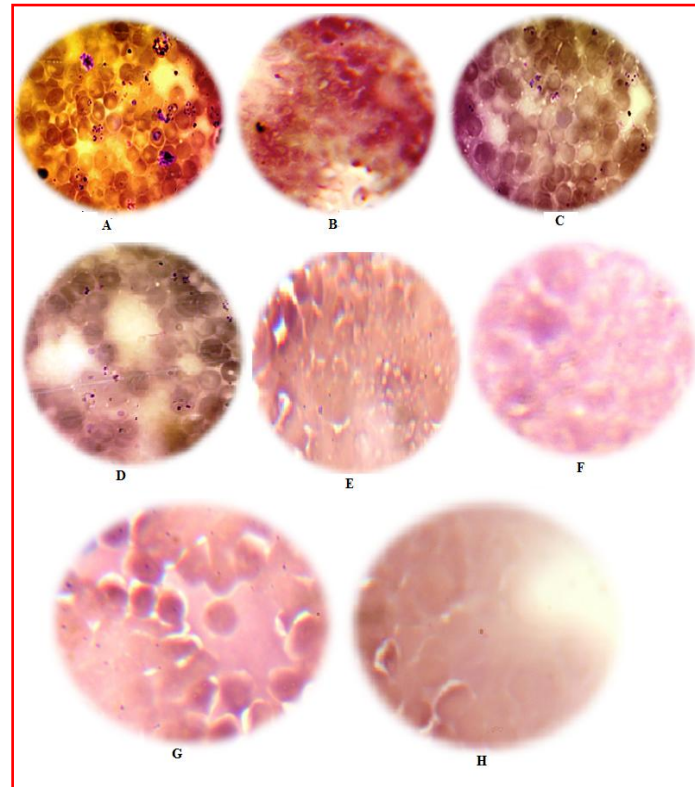

Figure 3(A-H): Showing schizonts maturation of P. falciparum in antimalarial sensitivity test for chloroquine (A-3D7 P. falciparum control strain without chloroquine and from B to H contains drug in different concentrations and patient samples).
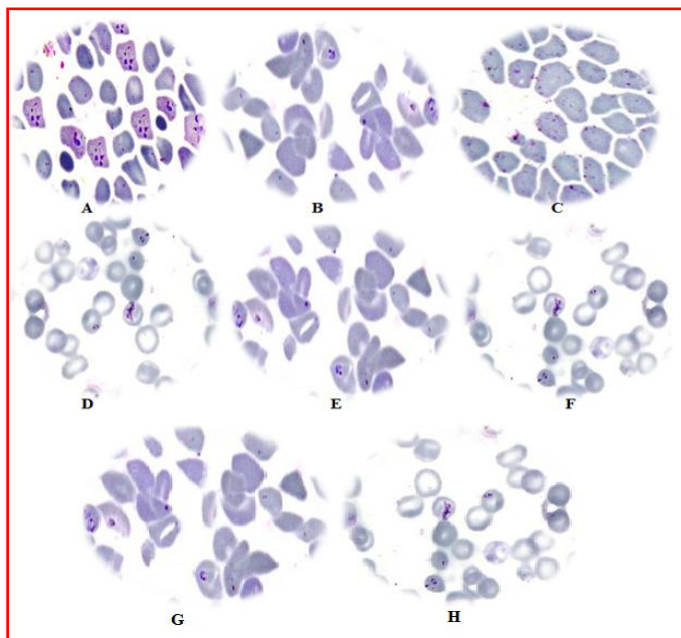
Figure 4(A-H): Showing schizonts maturation of P. vivax in antimalarial drug sensitivity test for chloroquine (A- P. vivax control strain without chloroquine and from B to H contains drug in different concentrations and patient samples).
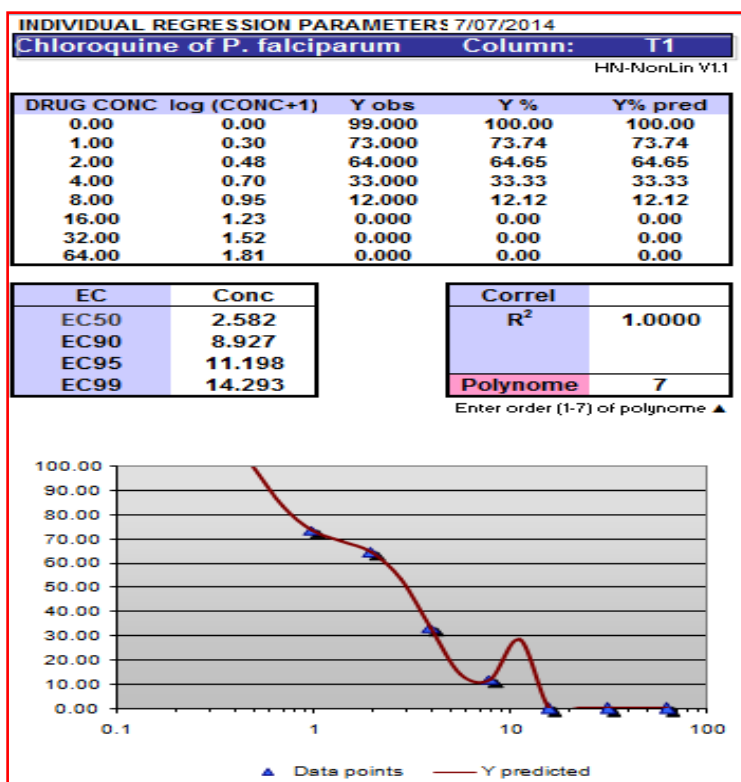

Figure 5: Showing regression parameter of antimalarial drug concentration.

III.   Results

Drug sensitivity testing for 22 patients samples was carried out using control strain of Plasmodium falciparum (3D7) against various dilution of following antimalarial drugs: 1) Chloroquine 2) Mefloquine 3) Quinine 4) Artesunate 5) Arteether 6) Primaquine 7) Sulfadoxine / pyrimethamine and 8) Amodiaquine. Drug resistance of Plasmodium falciparum to chloroquine was found to be 15/22 (68.18%). Amodiaquine was 4/22 (18.18%) resistance, Sulfadoxine / pyrimethamine was 3/22 (13.64%). No resistance was seen in Artesunate, Arteether and Mefloquine.

Drug sensitivity testing for 22 patients samples was carried out using control strain of Plasmodium vivax positive control against various dilution of following antimalarial drugs: 1) Chloroquine 2) Mefloquine 3) Quinine 4) Artesunate 5) Arteether 6) Primaquine 7) Sulfadoxine / pyrimethamine and 8) Amodiaquine. Drug resistance of Plasmodium vivax to chloroquine was found to be 5/22 (27.73%). Primaquine was resistance 3/22 (13.64%) only. No resistance was seen in Artesunate, Arteether amodiaquine, quinine, Mefloquine and Sulfadoxine / Pyrimethamine.

Table 1: Showing sensitivity pattern of Plasmodium falciparum.

Antimalarial drugs	P. falciparum (n=22)	
	Sensitive	Resistance
Chloroquine	7	15
Quinine	22	0
Mefloquine	22	0
Arteether	22	0
Artesunate	22	0
Amodiaquine	18	4
Sulfadoxine / Pyrimethamine	19	3

Table 2: Showing sensitivity pattern of Plasmodium vivax

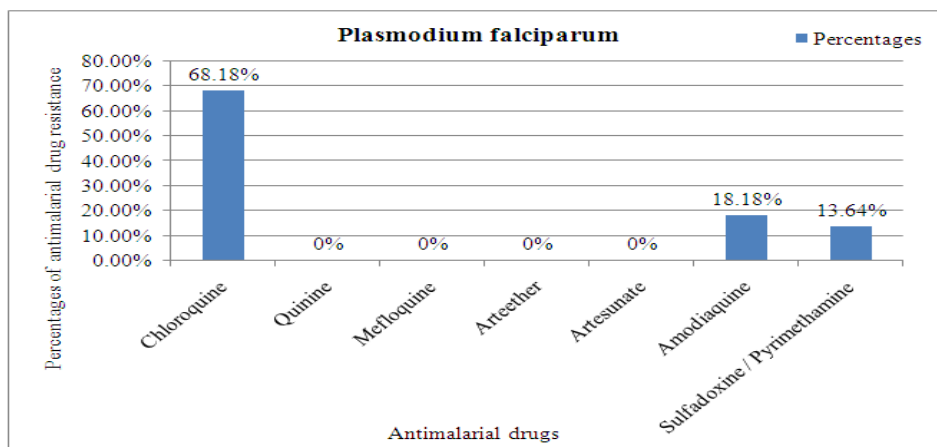| Antimalarial drugs	P. vivax (n=22)	
	Sensitive	Resistance
Chloroquine	17	5
Quinine	22	0
Mefloquine	22	0
Arteether	22	0
Artesunate	22	0
Amodiaquine	22	0
Sulfadoxine / Pyrimethamine	22	0
Primaquine	19	3

Figure 3: Showing percentages of drug resistance pattern of P. falciparum.
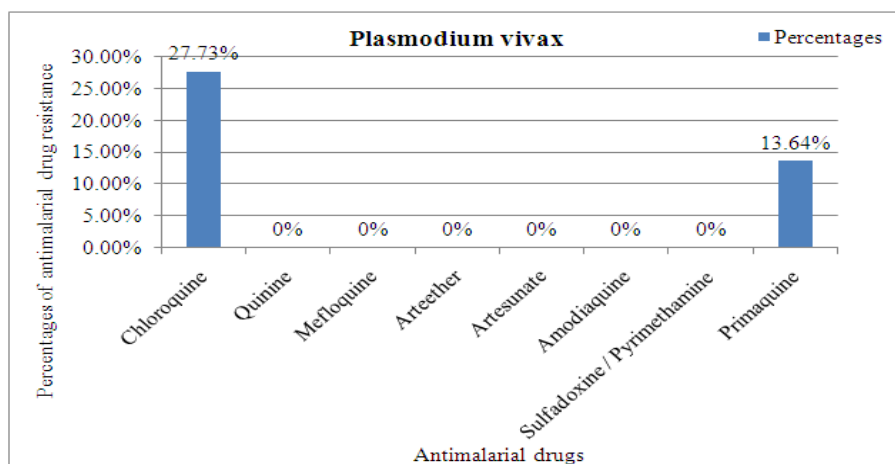

Figure 4: Showing percentages of drug resistance pattern of P. vivax

IV.   Discussion

The present study was undertaken to establish and standardize the test procedures for cultivation of malarial parasites and its drug sensitivity testing.

Antimalarial drug sensitivity is done in very few laboratories across the world specially for research purposes. The reason being difficulty to maintain the sterility of medium and growth of parasites. We performed antimalarial drug sensitivity testing of patients (44) samples. 22 P. falciparum and 22 P. vivax. We found chloroquine resistance in P. falciparum was quit high at 68.18% (15/22). There was growth of malarial parasite (mature schizont formation) in 8 pmol / l drug concentration indicating resistance to chloroquine. This finding is close to Shrivastava SK et al.[18] who reported resistance of 86.96% of P. falciparum to chloroquine in Assam area. Fatima Shujatullah et al.[13] from Aligarh and Anupkumar R. Anvikar et al.[19] from New Delhi reported lower values i.e. 24.07% and 44.4% respectively.

The explanation of high drug resistance to chloroquine could be that chloroquine is given to all febrile patients in endemic areas as prophylactic measure. This continuous indiscrimate widespread use of chloroquine must have led the malarial parasite to develop some mechanisms to overcome effect of chloroquine. Chloroquine resistance has been reported by workers in other countries also - Peletiri I et al.[20] (Nigeria) 88.9%, Oyedeji Segun Isaac et al.[22] (Nigeria) 80%, Halima Kaddouri et al.[31] (Mali) 60-79%, Pascal Ringwald et al.[21] (Cameroon) 62.18%, Olasehinde GI et al.[25] (Nigeria) 51% and Didier Menard et al.[30] (Central Africa) 37%. Resistance to amodiaquine was found to be 18.18% (4/22). Anupkumar R. Anvikar et al.[19] from New Delhi reported resistance of 25%. Workers from other countries reported resistance to amodiaquine ranging from 13% by Olasehinde GI et al.[25] (Nigeria) to 44.4% by Peletiri I et al.[20] (Nigeria). Resistance to sulfadoxine / pyrimethamine was found to be 13.64% (3/22). Didier Menard et al.[30] (Central Africa) and Olasehinde GI et al.[25] (Nigeria) reported resistance of 38.3% and 5% respectively. No resistance was found in our study for artesunate, arteether (artemisinin), mefloquine and quinine. Anupkumar R. Anvikar et al.[19] from New Delhi also reported similar finding. Shrivastava SK et al.[18] (Assam, India) and Fatima Shujatullah et al.[13] (Aligarh, India) did not mention or study the resistance to artemisinin. No resistance to artemisinin was reported by workers from other countries. Didier Menard et al.[30] (Central Africa) however reported resistance to mefloquine 1.6%. Multidrug resistance (drug resistance to 3 or more antimalarial drugs) was not found in our study. Didier Menard et al.[30] (Central Africa) also reported similar findings.

Other workers did not comment on multidrug resistance (MDR). Antimalarial drug sensitivity testing on Plasmodium vivax showed chloroquine resistance of 27.73% (5/22). Chehuan YF et al.[23] (Brazil) has reported chloroquine resistance in P. vivax of 10.7% and mefloquine 6.4%. We have found 13.64% (3/22) resistance to primaquine for P. vivax. There was no mention off primaquine drug sensitivity testing by other workers.

V.   Conclusion

Sensitivity to antimalarial drugs was performed on 44 patient samples. 22 were P. falciparum and 22 were P. vivax. This number was decided upon as in one microtitre plate, we can perform drug sensitivity testing for 11 patient samples and one control. 68.18% of P. falciparum blood samples showed resistance to chloroquine. Resistance was also detected for amodiaquine (18.18%) and sulfadoxine / pyrimethamine (15%). No resistance was detected for artemisinin, mefloquine and quinine. P. vivax showed 27.73% resistance to chloroquine and 13.64% resistance to primaquine. No resistance was found for other antimalarial drugs. Chloroquine resistance has developed because of indiscrimate use of this drug. Proper diagnosis of malaria and drug sensitivity testing should be done routinely to prevent emergence of resistance to antimalarial drugs. Monotherapy should be avoided and combined drug therapy will reduce chances of emergence of drug resistance.

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