IN VITRO SENSITIVITY OF *PLASMODIUM FALCIPARUM* TO SULFADOXINE AND PYRIMETHAMINE

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INTRODUCTION

Sulfadoxine-pyrimethamine (S-P) combination was highly effective in treatment of chloroquine-resistant *Plasmodium falciparum* in Thailand. The cure rates obtained in falciparum malaria with 1000 mg sulfadoxine and 50 mg pyrimethamine were 89.5% (Harinasuta *et al.*, 1967) and 82% (Segal *et al.*, 1975), and 85%with 1500 mg sulfadoxine and 75 mg pyrimethamine (Doberstyn *et al.*, 1976).

In recent years, this combination has been reported to be less effective against *P. falciparum* in Thailand. From 1978 to 1980, the cure rate dropped from 32% to 14% (Bunnag *et al.*, 1980), and in children dropped from 76.7% during 1971-1977 to 7.4% in 1980 and 11.1% in 1981 (Chongsuphajaisiddhi *et al.*, 1979; Chongsuphajaisiddhi and Sabchareon, 1981).

Rieckmann *et al.*, (1968) described a simple *in vitro* technique for differentiation of drug-resistant and drug-sensitive strains of *P. falciparum*, and determination of drug susceptibility of *P. falciparum* was by an *in vitro* microtechnique (Rieckmann *et al.*, 1978). In 1980, Yisunsri and Rieckmann assessed the value of the *in vitro* microtechnique using parasites obtained from continuous culture. It was recognized that RPMI 1640 medium was not suitable for the sensitivity test of sulfa drugs (Desjadins *et al.*, 1979).

Brockelman and Tan-ariya (1982) demonstrated that the unsuitability of RPMI was due to its para-amino-benzoic acid (PABA) component. The growth factor in such high concentration (1 mg/liter) competed with the uptake of the drug. As an alternative Waymouth MB 754/1 medium was used. This new medium, supplemented with 10% human serum had a final PABA concentration between 25 and 60 ng/ml medium (Tan-ariya and Brockelman, 1983).

This study was carried out to assess the *in vitro* susceptibitity of *P. falciparum* to sulfadoxine and pyrimethamine in three types of media; Waymouth MB 752/1 alone, with 10% human serum and RPMI 1640.

MATERIALS AND METHODS

Heparinized or defibrinated blood specimens were collected from 39 pediatric patients with falciparum malaria admitted to the Hospital for Tropical Diseases, Bangkok, during August 1981 to January 1982. The collections were made before administration of antimalarials.

Drug susceptibility of *P. falciparum* to sulfadoxine, pyrimethamine and the combination was tested. Sensitivity to both sulfadoxine and pyrimethamine alone and a combination of sulfadoxine with pyrimethamine was also studied.

The three preparations of growth media were used; Waymouth MB 752/1 alone and plus 10% human serum, and RPMI 1640.

The assay for drug sensitivity was by *in vitro* microtechnique (Rieckmann *et al.*, 1978; Yisunsri and Rieckmann, 1980). Fifty µl of 10% blood suspension in growth medium was added to each well of both control and drug treated wells of flat bottomed tissue culture plates. The plate was covered with a plastic lid, agitated briskly to ensure that the dried drug has completely dissolved. Then, the culture plate was placed in a candle jar and incubated at 38°C for 48 hours. After incubation, the medium layer in each well was removed as much as possible by means of an ordinary capillary tube fitted with a rubber bulb. One thick smear made from the remaining red blood cells in each well was dried overnight, stained for 30 minutes with Giemsa stain (2% solution in 7.1 pH phosphate buffer) and examined under a light microscope.

From each specimen containing the drug, the number of normal and/or abnormal schizonts in 200 asexual parasites were counted. The results were expressed as a percentage of the mean number of schizonts per 200 asexual parasites in the three control specimens.

MIC_1 was the minimal concentration that inhibited normal schizont formation. The end point was the first drug concentration in which no normal schizont was observed.

MIC_2 was the minimal concentration that inhibited schizont formation. The end point was the first drug concentration in which there was no appearance of schizont.

RESULTS

Sixty-five blood specimens were collected from 39 falciparum malaria pediatric cases admitted to the Hospital for Tropical Diseases. The success in *in vitro* study in Waymouth alone, Waymouth with 10% additional human serum, and RPMI were 87.2, 87.2 and 64.1% respectively.

Table 1 and 2 shows susceptibility of *P. falciparum* to sulfadoxine, pyrimethamine

Table 1

MIC_1 (n mol per 5 µl blood) of sulfadoxine (S), pyrimethamine (P) and sulfadoxine-pyrimethamine of *P. falciparum*.

Drug	Waymouth		Waymouth plus serum		RPMI	
	No. cases	MIC_1	No. cases	MIC_1	No. cases	MIC_1
S	25	8-240* 143.92** 120***	24	10-240 142.92 120	20	20-240 155.0 140
P	35	0.002-2.0 0.229 0.1	34	0.008-6.0 0.582 0.25	24	0.008-4.0 1.980 2.0
S-P	24	0.2/0.001-120/0.6 15.312/0.77 8/0.04	34	0.2/0.001-120/0.6 18.665/0.093 10/0.05	21	0.4/0.002-300/1.5 63.352/0.317 40/0.2

* Range
** Mean
*** Median

Table 2

MIC_2 (n mol per 5 µl blood) of sulfadoxine (S), pyrimethamine (P) and sulfadoxine-pyrimethamine of *P. falciparum*.

Drug	Waymouth		Waymouth plus serum		RPMI	
	No. cases	MIC_2	No. cases	MIC_2	No. cases	MIC_2
S	25	80- > 240* 120**	24	120- > 240 160	20	40- > 240 200
P	35	1.0- > 8.0 4.0	34	0.5- > 8.0 4.0	24	0.05- > 8 4.0
S-P	24	4/0.02- > 300/1.5 140/0.7	24	20/0.1- > 300/1.5 160/0.8	21	10/0.05- > 300/1.5 120/0.6

* Range
** Mean

and the combination in the three different media.

MIC_1 of various drugs except sulfadoxine obtained from Waymouth and RPMI showed significant difference ($p < 0.001$-$p < 0.01$) but MIC_2 of all drugs were not significantly different ($p > 0.05$).

MIC_1 of all drugs except sulfadoxine obtained from Waymouth plus serum and RPMI also showed significant difference ($p < 0.001$-$p < 0.05$) while MIC_2 of all drugs were not significantly different ($p > 0.05$).

MIC_1 as well as MIC_2 of all drugs obtained from Waymouth alone, and plus serum showed no significant difference ($p>0.05$) but both values in the latter medium were slightly higher. Both MIC_1 and MIC_2 of sulfadoxine or pyrimethamine alone was significantly higher than when either drug was combined with the other ($p < 0.001$-$p < 0.05$). No interaction appeared between media and drugs ($p > 0.05$).

There was a significant difference between MIC_1 and MIC_2 of sulfadoxine in all media ($p<0.001$) while no significance was observed for pyrimethamine ($p > 0.005$). MIC_1 and MIC_2 of the combination in Waymouth alone, and plus serum were not significantly different ($p > 0.05$) while the ones in RPMI showed significant difference ($p < 0.01$).

DISCUSSION

The finding that MIC_1 in RPMI was higher than in Waymouth alone and with serum confirmed the study of Brockelman and Tanariya (1982) that PABA presence in RPMI interfered with the sensitivity of the parasite to sulfadoxine (PABA antagonist) and pyrimethamine (dihydrofolate reductase inhibitor).

MIC_1 as well as MIC_2 in Waymouth plus serum was slightly higher than in Waymouth alone but without any significant difference. This is because both preparations contained

PABA, 5% patients' sera, while the former contained more PABA, 10% additional human serum.

Waymouth alone is rich enough for short term cultivation of the parasites, and is considered more suitable for *in vitro* microtechnique to test the sensitibity of *P. falciparum*. Addition of 10% normal human serum to this medium did not show any advantage, most likely it will interfere with the drug uptake of parasites. This study also confirmed that sulfadoxine and pyrimethamine show synergistic activity to each other. Although the proportion of sulfadoxine to pyrimethamine in the ingested S-P tablet is 20 to 1, in this experiment it was 200 to 1 according to their serum levels. Further studies are needed to elucidate the action of sulfadoxine and pyrimethamine on the malarial parasites.

Chongsuphajaisiddhi *et al.*, (1981a) reported that treatment failure of falciparum malaria with quinine might occur in cases infected by parasites with high MIC in which serum quinine concentration could not be maintained above the MIC longer than 6 days after the commencement of quinine therapy.

Fig. 1 shows the relationship between mean MIC_1 and MIC_2 ofsulfadoxine in combination with pyrimethamine of certain cases from this study and its serum concentration (from the study of Chongsuphajaisiddhi *et al.*, 1981b: pers.comm.). It was observed that the serum concentration could not be maintained above the MIC_2 in Waymouth alone, or plus serum longer than 6 days after sulfadoxine and pyrimethamine therapy. The maximal serum level was markedly lower than the MIC_1 in RPMI and the MIC_2 in all media. This might be one reason for treatment failure of falciparum malaria with S-P. The MIC_1 was lower than the maximum serum level of the drug while the MIC_2 was markedly higher indicating that MIC_1 might be more appropriate than MIC_2 for clinical study.

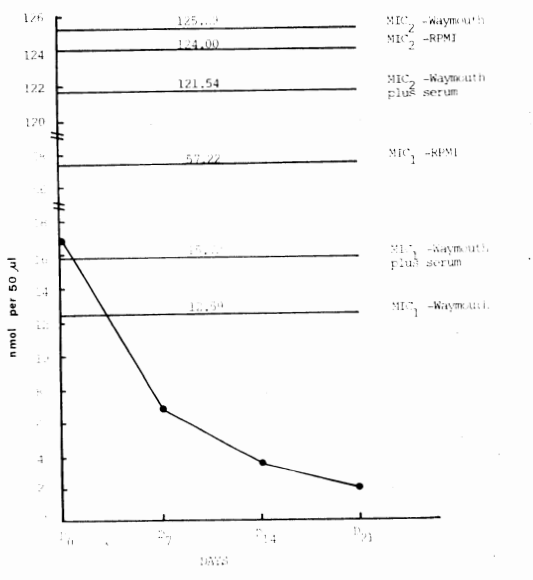

Fig. 1—Relationship between MIC_1, MIC_2 of sulfadoxine (S) plus pyrimethamine (P) and serum level of sulfadoxine after S-P combination therapy.

Yisunsri and Rieckmann (1980) studied on the susceptibility of a chloroquine-resistant strain of *P. falciparum* to pyrimethamine by *in vitro* microtechnique of Rieckmann *et al.*, (1980), using RPMI as growth medium. The strain originally obtained from a patient in Thailand, had been cultured by method of Trager and Jensen (1976). In all of their experiments (both 24 and 48 hours of incubation), normal schizonts were entirely replaced by abnormal schizonts when samples contained 3.0 n mols of pyrimethamine per ml of 40% erythrocyte suspension which is equivalent to the concentration of 0.015n mol per 5 µl blood. This concentration was within the range of MIC_1 (0.008-4.0 n mol per 5 µl blood) of this study.

Beales *et al.*, (1980) studied the pyrimethamine sensitivity of *P. falciparum* isolated from Kampuchean refugees and indigenous Thais by the same *in vitro* microtechnique using RPMI as growth medium. The study revealed range (mean) of MIC_1 in Kampuchean and

Thai group as 0.256- 1.024 (0.266) and 0.064- 0.512 (0.272) nmol per 5 µl blood respectively, which are within the range of this study (0.008-4.0 nmol per 5 µl blood).

Thaithong and Beales (1981) also reported the pyrimethamine sensitivity of ten Thai isolates of *P. falciparum* from three endemic areas in Central Thailand: Kanchanaburi, Sriracha and Phra Phutthabat using a method similar to those of Nguyen-Dinh and Trager (1978) and Richards and Maples (1979) with RPMI plus serum as the growth medium. They maintained in culture for at least 10 cycles (20 days). The parasite growth was inhibited by pyrimethamine at 1×10^{-7} - 5×10^{-5} M which is equivalent to 0.005 - 2.5 nmol per 5 µl blood in this study, and within the range of MIC_1 and MIC_2 (0.008 - 4.0 nmol per 5 µl blood and 0.05-8.0 n mol per 5 µl blood).

The sensitivity of Thai strain of *P falciparum* to sulfadoxine and pyrimethamine was also studied by Brockelman and Tan-ariya (1982) using two media, Waymouth and RPMI, plus additional human serum. The results read at 50 percent reduction of parasite multiplication rate were also within the range of MIC_1 of this study.

The exact comparison of the results of this study with those of Thaithong and Beales (1980) and Brockelman and Tan-ariya (1982) using the strain of *P. falciparum* from continuous culture is difficult, because PABA concentration was not standardized in the system used in this present study. The level of PABA-sulfadoxine competition was shown to be at 50 ng PABA/ml medium onward and PABA concentration beyond this can be reached easily when 15% human serum is added to Waymouth (Tan-ariya and Brockelman, 1983) However, the results of previous studies are within the range of MIC_1 of this study.

SUMMARY

An *in vitro* microtechnique of Rieckmann *et al.*, (1978) modified by Yisunsri and Rieckmann (1980) using 3 media; Waymouth, Waymouth plus 10% human serum, and RPMI was assessed to determine the sensitivity of *P. falciparum* to sulfadoxine, pyrimethamine and its combination.

The study confirmed the synergism between sulfadoxine and pyrimethamine. There was no interaction between media and drug tested. MIC_1 and MIC_2 of sulfadoxine in different media showed significant difference ($p<0.001$). No significant difference was observed in MIC_1 and MIC_2 of pyrimethamine in the three media used ($p>0.05$). For sulfadoxine-pyrimethamine combination, MIC_1 and MIC_2 in Waymouth alone and plus 10% human serum showed no significance ($p>0.05$) while in RPMI showed positive correlation ($p<0.001$). MIC_1 might be more applicable for clinical evaluation than MIC_2.

At present Waymouth medium with 5% patient serum, is considered to be the most suitable for testing sensitivity of malarial parasites.

REFERENCES

BEALES, P.F., YISUNSRI, L. and YAMOKYL, P., (1980). A study to determine appropriate antimalaria measures in areas of mass immigration in Eastern Thailand. WHO report.

BROCKELMAN, C.R. and TAN-ARIYA, P., (1982). *Plasmodium falciparum* in continuous culture : A new medium for the *in vitro* test for sulfadoxine sensitivity. *W.H.O. Bull., 60* : 432.

BUNNAG, D., HARINASUTA,T., SUNTHARASAMAI, P., MIGASENA, S., CHAREONLARP, P., VIRAVAN, C., VANIJANONDH, S. and RIGANTI, M., (1980). *In vivo* response

of *Plasmodium falciparum* to different regimes of anti-malarial drugs. Faculty of Tropical Medicine, Annual Report.

CHONGSUPHAJAISIDDHI, T. and SABCHAREON, A., (1981). Sulfadoxine-pyrimethamine resistant falciparum malaria in Thai children. *Southeast Asian J. Trop. Med. Pub. Hlth., 12* : 418.

CHONGSUPHAJAISIDDHI, T., SABCHAROEN, A. and ATTANATH, P., (1981a). *In vivo* and *in vitro* sensitivity of falciparum malaria to quinine in Thai children. *Ann. Trop. Paed., 1* : 21.

CHONGSUPHAJAISIDDHI, T., SABCHAREON, S., PUANGPARTK S. and HARINASUTA, T., (1979). Treatment of falciparum malaria in Thai children. *Southeast Asian J. Trop. Med. Pub. Hlth., 10* : 132.

DESJARDINS, R.E., CANFIELD, C.J., HAYNES, J.D. and CHULAY, J.D., (1979). Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrob. Agents Chemother., 16* : 710.

DOBERSTYN, E.B., HALL, A.P., VETVATANAPUBUL, K. and SONKOM, P., (1976). A single dose therapy of falciparum malaria using pyrimethamine in combination with diformyldapxone or sulfadoxine. *Amer. J. Trop. Med. Hyg., 25* : 14.

HARINASUTA, T., VIRAVAN, C. and REID, H.A., (1967). Sulphomethoxine in chloroquine-resistant falciparum malaria in Thailand. *Lancet, 1* : 1117.

NGUYEN-DINH, P. and TRAGER, W., (1978). Chloroquine resistance produced *in vitro* in an African strain of human malaria. *Science, 200* : 1397.

RICHARDS, W.H.G. and MAPLES, B.K., (1970). Studies on *Plasmodium falciparum* in continuous cultivation. I. The effect of chloroquine and pyrimethamine on parasite growth and viability *Ann. Trop. Med. Parasit., 73* : 99.

RIECKMANN, K.H., MCNAMARA, J.V., FRISCHER, H., STOCKERT, T.A., CARSON, P.E. and POWELL, R.D., (1968). Effects of chloroquine, quinine and cycloguanil upon the maturation of asexual erythrocyte forms of two strains of *Plasmodium falciparum in vitro. Amer. J. Trop. Med. Hyg., 17* : 661.

RIECKMANN, K.H., CAMPBELL, G.H., SAX, L.J. and Mrema, J.E.K., (1978). Drug sensitivity of *Plasmodium falciparum* : an *in vitro* microtechnique. *Lancet, 1* : 20.

SEGAL, H.E., CHINVANTHANANOND, P., LAIXUTHAI, B., PEARLMAN, E.J., HALL, A.P., PHINTUYOTHIN, P., NA-NAKORN, A. and CASTANEDA, B.F., (1975). Comparison of diaminodiphenylsulphone-pyrimethamine and sulfadoxine-pyrimethamine combinations in the treatment of falciparum malaria in Thailand. *Trans. Roy. Soc. Trop. Med. Hyg., 69* : 139.

TAN-ARIYA, P. and BROCKELMAN, C.R., (1983). *Plasmodium falciparum* : Variations in p-aminobenzoic acid requirements as related to sulfadoxine sensitivity. *Exp. Parasit., 55* : 364.

THAITHONG, S. and BEALES, G.H., (1981). Resistance of ten Thai isolates of *Plasmodium falciparum* to chloroquine and pyrimethamine by *in vitro* tests. *Trans. Roy. Soc. Trop. Med. Hyg., 75* : 271.

TRAGER, W. and JENSEN, J.B., (1976). Human malaria parasites in continuous culture. *Science, 193* : 674.

YISUNSRI, L. and RIECKMANN, K., (1980). *In vitro* microtechnique for determining the drug susceptibility of cultured parasites of *Plasmodium falciparum. Trans. Roy. Soc. Trop. Med. Hyg., 74* : 809.