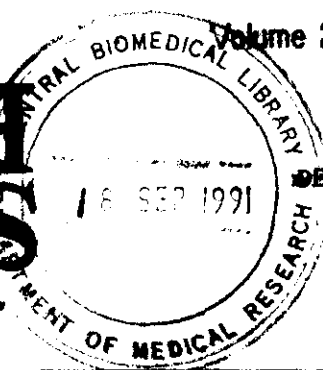


MYANMAR HEALTH SCIENCES RESEARCH JOURNAL



Volume 2, Number 3 December 1990

DEPARTMENT OF MEDICAL RESEARCH

No. 5, Ziwaka Road,
Yangon 11191, Myanmar

Efficacy of anti-viper venom administered intramuscularly in mice <i>Aye-Thán & Saw J. Tha</i>	107
Disposition kinetics of intramuscular quinine in Myanmar adult patients with falciparum malaria <i>Cho-Cho-Myint, Tin-Shwe & Pe-Than-Myint</i>	111
Serum uric acid level as predicting sign of preeclampsia <i>Ye-Tint-Lwin, Lilian Po & Hnin-Hline</i>	114
Vero cell cytotoxic <u>Escherichia coli</u> isolated from children with diarrhoea <i>Mar-Mar-Nyein, Kyu-Kyu-Mar, Tin-Aye & Hayashi H.</i>	118
Detection of <u>Escherichia coli</u> enterotoxin from stool samples directly by Bead ELISA method <i>Mar-Mar-Nyein, Oku Y., Than-Saw, Tin-Aye & Taketa T.</i>	130
The effect of chemical agents on contaminated water <i>Khin-Nwe-Oo, Zin-Zin-Moe, Aung-Myo-Han & Tin-Aye</i>	135
Effect of alum on bacteria in water <i>Khin-Nwe-Oo, Aung-Myo-Han & Tin-Aye</i>	138
Some variations in membrane proteins of erythrocytes obtained from subjects with thalassaemia trait and G-6-PD deficient genes <i>Myint-Oo, Myat-Phone-Kyaw & Thein-Than</i>	144
"DMR-Larval trap" for possible control of <u>Aedes aegypti</u> (L), the principal vector of DHF in Yangon <i>Anthony Sebastian & Myat-Myat-Thu</i>	152
Lepromin reaction in patients with falciparum malaria <i>Tin-Shwe & Pe-Than-Myint</i>	157

Editor-in-Chief

Prof. May May Yi

Editor

Prof. Myint Lwin

Associate Editors

U Hla Pe Dr. Than Swe
 Dr. Thein Hlaing Dr. Saw J. Tha

Managing Editor

Dr. Myo Thein

Editorial Board

Dr. Aung Htun Thet (DPS)	Dr. Myint Lwin (DMR)
U Aye Kyaw (DMR)	Prof. Myint Thein (EENT)
U Ba Aye (DMR)	Dr. Myo Myint (Ortho)
Dr. Franco Tin (DOH)	Prof. Myo Thwe (IM-2)
Prof. Hla U (IM-2)	Dr. Nyunt Win (Neuro)
Prof. Hta Kyu (IM-1)	Saw Marcus Win (VBDC)
Dr. Khin Maung Win (DMR)	Mr. A.A. Sebastian (DMR)
Prof. Kin Ma Gyi (IM-1)	Prof. R. Sein Dwe (IM-2)
Dr. Ko Ko Gyi (DCPT)	Dr. Shwe Tin (Cardiac)
Dr. Kyaw Lwin (DOH)	Dr. Soe Thein (DMR)
Prof. Kyaw Tint (IM-1)	Dr. Thein Maung Myint (DMM)
Col. Kyaw Win (MOD)	Prof. Thein Nyunt (IM-1)
Prof. Kyi Kyi (YU)	Dr. Thein Oke Kyaw Myint (IM-2)
Prof. Kyi Kyi May (IM-1)	Prof. Thein Thein Myint (IM-2)
Dr. Malar Maung (IM-1)	Prof. Thin Thin Hlaing (IM-2)
Prof. Maung Maung Nyo (IM-1)	Dr. Tin Aye (DMR)
Dr. Maung Maung Oo (DMR)	Dr. Thyra Po (DOH)
Prof. Mya Mya (IM-1)	Dr. Tun Pe (DMR)

Consultant Editors

Prof. Han Tin (YU)
 Associate Prof. Thi Ha (Inst. Ed.)

Publisher

U Aye Kyaw

Editorial Managers

Dr. Khin Maung Win
 U Khin Maung Naing

Production Managers

Dr. Tin Shwe
 Dr. Tin Nu Swe

Production Editors

U Aye Kyaw
 U Kyi Thaung

Efficacy of anti-viper venom administered intramuscularly in mice

Aye-Than & Saw J. Tha

Pharmacology Research Division
Department of Medical Research

The acute toxicity of Russell's viper venom of Myanmar variety was determined, - the LD 50 on mice being 300 µg/kg (range: 211-420 µg/kg), intraperitoneally. Following the envenomation with 1.6 mg/kg, the anti-viper venom administered 1 and 10 ml/kg intramuscularly had markedly reduced ($p < 0.001$) the mortality in mice to 30 and 70 per cent respectively, when compared with that of the placebo-treated group. However, the intramuscularly administration efficacy was less than when applied intravenously. It is projected to employ approximately 6-folds of the intravenous dosage to achieve the equipotency.

INTRODUCTION

Workers and farmers in the countryside are the most vulnerable victims to the Russell's viper bites(1). The incidence, being of emergency nature, occurred mostly in the outlying areas distant to the existing peripheral health centres and hospitals where competent health personnel could institute prompt medical intervention. The intravenous anti-viper venom (AVV) administration, even though is most ideal in combating the emergency, has a limitation in applying the process thereof. An alternative relatively applicable easy route of administration, being an intramuscular one, could resolve this limitation. Such a mean was claimed applicable with the AVV produced at the Myanmar Pharmaceutical Industry. However, the firm could not provide concrete scientific data regarding the efficacy of the recommended intramuscular dosage of 20-30 ml. Knowledge on assurance on the dosage for the efficacy is most important for the circumstance. To that end, the present study aimed at finding an answer, employing experimental model while circumventing any ethical issue.

MATERIALS AND METHODS

a. Determination of acute toxicity of viper venom

The acute toxicity, in terms of the LD50, of Russell's viper venom of Myanmar variety when applied intraperitoneally was determined(2). Adult Swiss mice, of both sexes, weighing 22.0 ± 0.6 g, were housed in cages of fives. A total of 80 animals were intraperitoneally injected with the venom, 50-1600 µg/kg. The route was chosen for its simulating nature of distribution to that of the intravenous route, technique-wise easily applicable and producing consistent results. The term "intravenous" is thus synonymous to "intraperitoneal" throughout the text.

b. Determination of the effects of anti-viper venom applied intramuscularly and intravenously

Adult Swiss mice of both sexes were used. The envenomation dose of viper venom employed was 1.6 mg/kg, approximately 5-folds of the LD50 already predetermined. After a time

interval of 10-15 minutes, various doses of AVV undiluted was administered either intravenously or intramuscularly. The control group was, however, administered with only physiological saline instead as a second injection. Following the latter injection, each mouse was observed for death within 24 hours and the time recorded. A comparison was made between the various groups tested for the time-course as well as mortality.

The experiment was repeated using different concentrations of the AVV diluted in physiological saline, being 1/10 and 3/10. The dosage of AVV injected was 0.01 ml/g body weight and was administered either intravenously or intramuscularly. A total of 90 mice divided into three groups were tested, and data compiled and analysed statistically.

RESULTS

a. Acute toxicity of viper venom intraperitoneally

The LD50 of viper venom on mice administered intraperitoneally was found to be 300 µg/kg (range: 211-426 µg/kg).

b. Effects of anti-viper venom applied intramuscularly and intravenously

The effectiveness of AVV administered intramuscularly and intravenously in mice are shown in Figure 1. The lower dose of AVV was found to save the victims to 30 per cent when employed intramuscularly. However a better result was obtained when applied intravenously, being up to 60 per cent. With the higher dose of 3/10 dilution employed, both the intramuscular and the intravenous routes of administration produced a better survival rate, the results being 30 and 80 per cent respectively. In the control group, only 10 per cent of mice survived. The intramuscularly applied AVV was signifi-

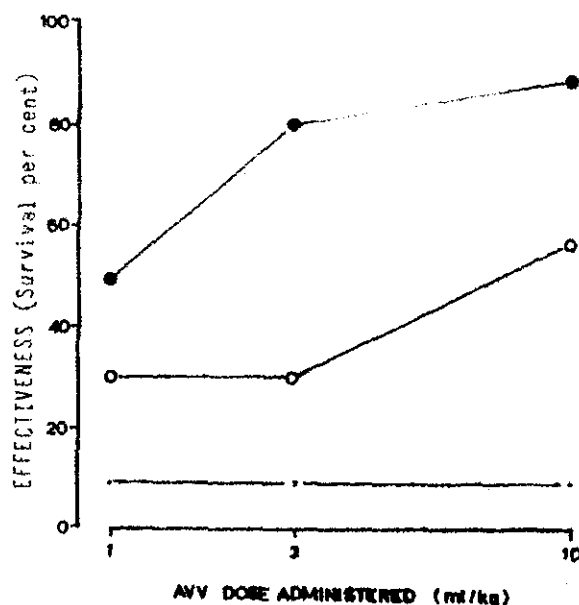


Fig. 1. Effectiveness of anti-viper venom administered by different routes in mice.

The response is expressed in terms of percentage of animal envenomated with viper venom 1.6 mg/kg, intraperitoneally, remained alive for a duration of 24 hrs in various groups of treatment, namely, control (•), intramuscular (○) and intravenous (●).

cantly less effective than that of the intravenous ($p < 0.05$). The undiluted AVV administered intramuscularly could save the victims to 70 per cent, but more animals up to 90 per cent had been saved when it was applied intravenously. Death rate in the control group remained the same. However, there was no significant difference between the intramuscularly employed group and that of the intravenously. Calculating on the basis of its *in vitro* neutralizing potency, it was found that the various concentrations of AVV of 1/10, 3/10 and 1 to be equivalent to 6-, 18- and 60-folds, respectively.

DISCUSSION

The execution of the present investigation necessitates a knowledge on definite killing dose of viper venom which is imperative. In Myanmar, the closest information on mice regarding the LD50 had been 250 µg/kg when applied subcutaneously(3) and 5.2[±]1.5µg per animal administered intravenously(4). A controversial report of the LD50 for the intravenous route were 80 µg/kg by Russell and Puffer(5) to 140 µg/kg by Ohsaka and co-workers(6). However, the potency of the Russell's viper venom investigated by the latter which was obtained from Los Angeles, California, USA, may be quite different from the one studied by Russell and Puffer(5). No such report has been available yet for the intraperitoneal route. The present work had enabled to yield information regarding the intraperitoneal route of administration that the LD50 was found to be 300 µg/kg, which is relatively higher than those of intravenous values reported elsewhere(5,6) but comparable to the intravenous result obtained on the local species(3). It therefore had confirmed the application of intraperitoneal route as equivalent to that of the intravenous.

Detailed analysis of the result revealed that further dilution of the concentration of the AVV produced no difference in its effectiveness, where the route of administration is concerned. Such a finding could be due to the anti-viper venom being in excess in combating the sub-minimal challenging dose of the venom. It was found that the intramuscular route of administration, when applied in a projected acceptable dose range, could effectively neutralize the majority of the envenomation. It was also suggestive the intramuscular route of administration could safely be employed in viper bite victims, even though less effective than when employed intravenously on a similar dose basis. The extent for intramuscular route of administration was 56.5 per cent as effective as the intravenous

route of administration. However, to obtain a similar efficacy it should be required to employ a larger amount of AVV. The dosage to be recommended is 5.9[±]0.2 times that of the dose needed in the intravenous application (Table 1), the amount appeared clinically realistic. The dose could attain the effective AVV level of at least 30 µg/ml, on the basis of report on human(7), with assurance of saving the lives of most victims.

Table 1. Calculated comparative efficacy of anti-viper venom in mice

Survival percentage	Dosage employed to be equal in efficacy (ml) (i.m.)	Dosage employed to be equal in efficacy (ml) (i.v.)	Equivalent folds (i.m. to i.v.)
60	5.6	0.10	5.60
70	1.0	0.18	5.71
80	2.0	0.32	6.25
85	2.9	0.48	6.04
90	4.9	0.80	6.12
95	10.5	1.75	6.00
100	1000.0	170.00	5.88
Mean			5.90
± s.e.			± 0.22

REFERENCES

1. Myint-Lwin, Warrell, D.A., Phillip R.E. et al. Bites by Russell's viper (*Vipera russelli siamensis*) in Burma: Haemostatic, vascular and renal disturbances and response to treatment. *Lancet*. 1985;ii: 1259-1264.
2. Litchfield, J.J. & Wilcoxon, F. A simplified method of evaluating dose-effect experiment. *Journal of Pharmacology and Experimental Therapeutics*. 1949; 96: 99-113.
3. Myint-Myint-Than. Changes in the renal tubules in viper envenomation. 1977; M.Sc. (Thesis), Rangoon Arts & Science University.
4. Khin-Swe-Thet. Immunogenicity and haemorrhagic activities of Russell's viper venom. 1977; M.Sc. (Thesis), Rangoon arts & Science University.

5. Russell, F. E. & Puffer, H.W.
Pharmacology of snake venom. In:
Snake Venoms and Envenomation.
Milton, S.A. (Ed.), 1971; 87-97.
6. Ohsaka, A., Omori-Sato, T., Kondo
H. et al. Biochemical and
pathological aspects of haemorrhagic
principles in snake venoms
with special reference to Habu
(Trimeresurus flavoviridis) venom.

Mem. Inst. Butantan. Simp.
Internac. 1966; 33(1): 193-205.

7. Hla-Pe, Thein-Than, Maung-Maung-
Thwin et al. Circulating antivenom
levels in human subjects intramus-
cular administration of Russell's
viper antivenom. Abstracts of
Medical Research Congress (Myanmar).
1989; p. 3.

Accepted for publication 13 June 1990.

Disposition kinetics of intramuscular quinine in Myanmar adult patients with falciparum malaria

Cho-Cho-Myint, Tin-Shwe** & Pe-Than-Myint****

*Department of Pharmacology, Institute of Medicine (2), Yangon

**Clinical Research Unit (Cerebral and complicated Malaria),
Department of Medical Research

***Department of Medicine, Institute of Medicine (1), Yangon

Intramuscular quinine pharmacokinetic study was made on ten Myanmar adults who attended Thayarwady Civil Hospital for the treatment of falciparum malaria. The patients were given intramuscular quinine dihydrochloride (15 mg base/kg) body weight followed by another two doses of intramuscular quinine 7.5 mg per kg and oral quinine sulphate 7.5 mg three times daily till day-7. Plasma concentrations of quinine at various post-drug time intervals were assayed by the benzene extraction fluorescence (BEF) method(1). Plasma quinine concentrations until 6 hours after the loading dose were analysed for pharmacokinetic parameters. The level of quinine reached the minimum inhibitory concentration level within the first 1-2 hours of starting treatment. The drug was found to be well tolerated by the patients with absence of tissue necrosis at the site of injection.

INTRODUCTION

Quinine is the standard drug for severe falciparum malaria, resistant to chloroquine. Quinine, when given by I.V. route is reliable in bioavailability, has no local pain nor tissue necrosis and is therefore currently employed as intravenous infusion. However, this method is expensive and requires trained personnel for administration compared to intramuscular route. Despite the simple technique there has been no known reports regarding the pharmacokinetic information of intramuscular quinine in Myanmar patients. The present study, therefore aimed at seeing to the suitable form of intramuscular quinine in Myanmar adults and a regimen designed to achieve therapeutic non-toxic plasma quinine concentration based on drug pharmacokinetic profile.

PATIENTS AND METHODS

Among falciparum malaria patients attending the Thayarwady Civil Hospital during 1988 malaria season, 10 male patients, age of 21 to 38 years and weight of 41 to 55 kg, were studied. Half of the patients were highly parasitised as more than 5% of their erythrocytes were parasitised. Intramuscular quinine dihydrochloride in 1.5% benzyl alcohol was used. It was prepared specially by the Myanmar Pharmaceutical Industry; MPI, Yangon, Myanmar.

Patients were screened for the presence of quinine in urine by using Mayer Turnet test. Only those who were initially free from quinine were included in the study.

Regimen employed in the present study

was, an intramuscular initial loading dose of 15 mg/kg body weight followed at 8-hourly intervals by 7.5 mg/kg for another 2 doses. This was then followed by oral quinine sulphate 10 mg/kg tds till day-7.

Clinical data were recorded 6-hourly throughout the 7-days hospital stay for blood film for parasites, blood pressure, pulse rate, temperature and signs and symptoms of toxicity. ECG recordings were done immediately and at the day-3, day-7 and day-14 respectively.

Blood samples for plasma quinine were collected at various time intervals: 0, 1, 2, 4, 6, 8, 12, 24 hours on day-1, and at day-2, 3, 4 and 7. Plasma was separated immediately and stored at minus 20 degree centigrade until assayed by the BEF method, using the Turner Filter Fluorometer Model 111 (Sequoia-Turner Corporation, California).

Plasma quinine concentration until 6 hours after the initial loading dose were analysed for single dose pharmacokinetics.

Evaluation

Pharmacokinetic parameters were calculated namely, plasma half-life ($t_{1/2}$), concentration at zero time (C_0), maximum plasma concentration (C_{max}), elimination rate constant (K_{el}), apparent volume of distribution (aVd) and systemic clearance (Cl).

RESULTS

All patients responded well to the treatment. In 9 out of 10 patients, quinine level reached above the minimum inhibitory concentration (MIC) level of 5.7 mg/L for Myanmar patients (Myint-Lwin, personal communication) from the first hour and the effective concentration was well maintained (Fig. 1). The remaining one patient had his serum quinine level also above the MIC level from the second hour. Major

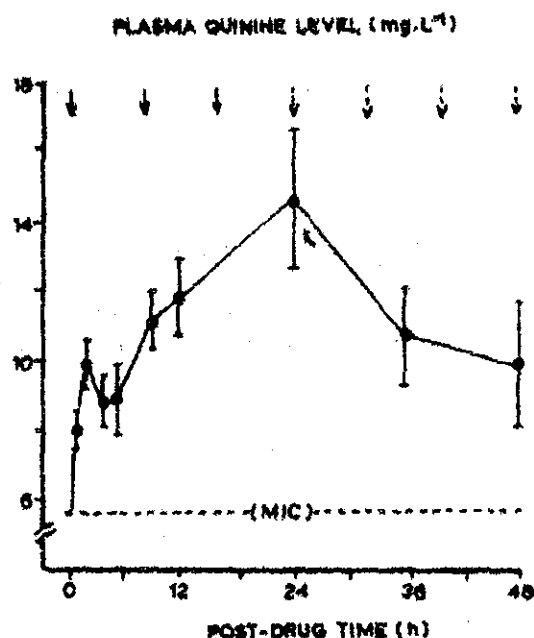


Figure 1. Time course response of quinine following quinine 15 mg/kg (intramuscular) and 7.5 mg/kg (orally) in Myanmar patients with falciparum malaria.

Each point represents the mean of 10 observations, together with the standard errors of the means shown as vertical bars. Each patient was administered with the drug both intramuscularly (thick arrow) and orally (dotted arrow).

Table 1. Pharmacokinetic profile of single intramuscular quinine 15 mg/kg in Myanmar patients with falciparum malaria

Parameters	Data
Plasma half-life($t_{1/2}$)	5.5 $\pm$ 0.3 h
Concentration at zero hour (C_0)	14 $\pm$ 0.9 mg l ⁻¹
Maximum concentration(C_{max})	10.5 $\pm$ 7 mg l ⁻¹
Time to reach maximum concentration (T_{max})	2.12 $\pm$ 0.1 h
Elimination rate constant (K_{el})	0.13 $\pm$ 0.08 h ⁻¹
Apparent volume of distribution (aVd)	1.53 $\pm$ 0.09 l Kg ⁻¹
Systemic clearance (Cl)	3.29 $\pm$ 0.2ml min ⁻¹ kg ⁻¹
Area under the curve (AUC)	6.34 $\pm$ 0.4 ug/ml

pharmacokinetic data are depicted in Table 1. None of the patients complained of local pain. Neither tissue necrosis nor abscess at the site of administration was observed.

DISCUSSION

The study found the intramuscular loading dose of quinine was simple to administer and effective therapeutically on all 10-patients. From the pharmacokinetic findings, quinine possessed therapeutic characteristics, namely, the MIC reached within the first 1-2 hours of starting the treatment. This method is less expensive and no special supervision is needed, in comparison to intravenous route. Plasma quinine concentration after intramuscular injection of quinine closely resembled that of quinine infusion followed by oral therapy(2). There had also been a report on a trial of intramuscular quinine on 32 patients with falciparum malaria(3). The latter workers had demonstrated that therapeutic serum concentrations can be reached and maintained by intramuscular injection of quinine dihydrochloride.

The lack of local pain, tissue necrosis and abscess at the site of intramuscular injection was noted in the present study and was most desirable for a patient's compliance and clinical management. It may be due to the addition of 1.5% benzyl alcohol in the new preparation, specially prepared by the MPI.

Hence intramuscular method of quinine may be recommended for patients with complicated falciparum malaria in the villages before they are transferred to the hospitals. The drug should also be

prepared by adding 1.5% benzyl alcohol as MPI has prepared. Therefore the drug form developed is most desirable to be employed with an added advantage of easier administration.

ACKNOWLEDGEMENTS

The authors are very grateful to the Rector Dr. U Myo-Thwe and Prof. Daw May-Winn-Aung-Khin of Institute of Medicine(2) for their kind permission to prepare this paper, Dr. Saw J. Tha, of the Department of Medical Research and Dr. U Aung-Naing, of the Department of Indigenous Medicine for close supervisions, and the MPI for supplying the modified preparation of quinine dihydrochloride injection.

REFERENCES

1. Chemotherapy of malaria. WHO series. 1986; 27: 198-199.
2. Pe-Than-Myint, Tin-Shwe, Tin-Aung & Kyin-Htwe. Controlled trial of initial slow I.V. quinine and conventional quinine infusion in the treatment of highly parasitised falciparum malaria in adult patients. South East Asian Journal of Tropical Medicine and Public Health. 1987; 18: 85-88.
3. Chingsulphajaisiddhi, T., Daurag, V., Patchovakessakul, V., Kittivattarakul, V., Dulyasakdi, B., Ridthimat, W. Treatment of falciparum malaria with intramuscular quinine in rural area of Thailand. South East Asia Journal of Tropical Medicine and Public Health. 1983; 14: 220-222.

Accepted for publication 13 June 1990.

Serum uric acid level as predicting sign of preeclampsia

Ye-Tint-Lwin, Lilian Po & Hnin-Hline

Physiology Department
Institute of Medicine 1 & 2

Serum uric acid levels were measured in 19 pregnant Myanmar women clinically diagnosed as preeclampsia, before any treatment was given. The subjects were in the period of 36 ± 2 weeks gestation and their ages ranged from 20 to 40 years. Out of 19 cases, 12 had mild preeclampsia and 7 had severe preeclampsia. All cases became normotensive and proteinuria ceased after puerperium. The serum uric acid levels of severe preeclampsia varied widely from 4.9 to 11.9 mg/dl while that of mild preeclampsia ranged from 3.4 to 7.9 mg/dl. The mean serum uric acid level of severe preeclampsia (9.3 ± 2.36 mg/dl) was significantly higher than that of apparently healthy pregnant women of the same age and gestational period. The mean value of mild preeclampsia (5.5 ± 1.38 mg/dl) was not significantly different from the normal. Therefore, a single uric acid level of a pregnant women is of little use as a predicting sign of preeclampsia.

INTRODUCTION

Since the usual clinical criteria (hypertension, edema and proteinuria) of preeclampsia are neither specific nor sensitive enough for the diagnosis, the development of a simple and sensitive biochemical test will be of great value to obstetricians. McFarlane(1) suggested that hyperuricemia was a useful diagnostic feature of early preeclampsia. It preceded the development of proteinuria and was the only simple investigation which could confirm the diagnosis in non-proteinuric preeclampsia. Therefore, estimation of serum uric acid has been done to assess whether it might be of help in diagnosing early preeclampsia.

MATERIALS & METHODS

The subjects were recruited from those attending antenatal clinics at the Defence Services General Hospital and

North Okkalapa General Hospital. They were 45 clinically normal, 20-40 year old, pregnant Myanmar women of 36 ± 2 weeks gestational period (as normal controls). No specific dietary restrictions were enforced but those who took fishegg, bamboo-shoot, meat and drugs on the day of sample collections were excluded from the study.

The preeclamptic patients were selected according to the criteria for preeclampsia laid down by Haynes(2) and degree of preeclampsia(mild or severe) was classified according to Weir(3).

All preeclamptic cases became normotensive and proteinuria ceased after puerperium.

After resting for 15 to 20 minutes, blood samples were taken from the antecubital vein in sitting position. Blood was allowed to clot in sterilized dry bottles and transported to the laboratory in a dark container and serum was stored overnight in a refrigerator at 4°C. Midstream specimen of random urine samples were collected

and tested for the presence of protein and sugar.

Serum uric acid level was measured by a simplified alkaline phosphotungstate assay for uric acid in serum (4). Blood pressure was measured with a mercury sphygmomanometer (5). Detection of protein in urine was performed by the sulphosalicylate test described in Varley, Gowenlock and Bell (6). Detection of sugar in urine was done by Benedict's qualitative test(6).

RESULTS

Table 1 shows serum uric acid levels in preeclampsia. Out of 19 cases, 12 cases (Sr. No. 1 to 12) had mild preeclampsia and 7 cases (Sr. No. 13 to 19) had severe preeclampsia. The serum uric acid levels of severe preeclampsia varied widely from 4.9 to 11.9 mg/dl while that of mild preeclampsia ranged from 3.4 to 7.9 mg/dl.

Table 1. Serum uric acid in preeclampsia
(n = 19)

No.	Age (year)	Weeks of gestation	Blood pressure mmHg	Edema	Protein - uria	Serum uric acid (mg/dl)
1	22	36	150/90	+	-	5.20
2	20	36	160/100	+	-	5.90
3	31	36	140/96	+	-	6.30
4	20	36	140/90	+	-	7.05
5	29	38	150/96	+	-	3.70
6	21	36	140/90	+	-	5.20
7	27	36	150/90	+	-	3.40
8	21	38	160/100	+	-	6.25
9	36	38	140/100	+	-	4.00
10	38	35	160/100	+	-	7.90
11	36	38	140/100	+	-	4.75
12	37	36	150/100	+	-	6.40
13	20	34	170/110	+	+	10.70
14	30	38	180/110	+	++	11.90
15	32	38	170/100	+	+	4.90
16	23	36	180/110	+	+	9.20
17	24	36	160/110	+	+	9.40
18	20	36	170/100	+	+	7.90
19	22	34	160/120	+	+	11.10

Table 2. Comparison of mean serum uric acid levels of normal pregnancy and preeclampsia at 36[±]2 weeks gestation

	Normal pregnancy	All preeclampsia	Mild preeclampsia	Severe preeclampsia	Significance of difference (p value)		
	(NP)	PE	mPE	sPE	NPvsPE	NPvs/mPE	NPvs sPE
Mean serum uric acid (mg/dl)	4.93	6.90	5.50	9.30	<0.01	>0.05	<0.01
S.D.	0.73	2.56	1.38	2.35	S	N.S.	S
Number of observations	45	19	12	7			
Mean gestational period (week)	36.13	36.36	36.58	36.00	>0.1	>0.1	>0.1
					N.S.	N.S.	N.S.

S = Significant
N.S. = Not Significant

Table 2 shows the comparison of the mean serum uric acid level between normal pregnancy and preeclampsia of same gestational period (36[±]2 weeks). There was a significant hyperuricemia in preeclampsia. When preeclampsia was divided into mild and severe cases and then compared to that of normal pregnant cases, the former was not significantly different but the latter was significantly higher than in normal pregnancy.

DISCUSSION

In the present study, the mean serum uric acid level in preeclampsia was 6.9[±]2.56 mg/dl. It was significantly higher than that of normal pregnancy of the same gestational period. This confirmed the well known fact that hyperuricemia during pregnancy was associated with preeclampsia (Slemon & Bogert 1917)⁷.

The mean serum uric acid level in mild preeclampsia was slightly higher than that of normal pregnant women in the same gestational period but the difference was not significant. This

finding was in agreement with the report of Seitchik (1953)⁸ which stated that the blood uric acid level in mild preeclampsia was within normal limits. Hayashi (1956)⁹ suggested that serum uric acid level was less reliable than uric acid clearance studies in preeclampsia. Fay, Bromham & Brooks et al 1985¹⁰ concluded that serum uric acid was of little value in predicting preeclampsia by showing that uric acid rose significantly only one week before delivery. Cadden & Stander (1933)¹¹, Langer & Fisher 1956¹², Pollak & Nettle 1960¹³, McFarlane 1963¹⁴ and Redman, Beilme & Bonnar 1976¹⁵ stated that the increased serum uric acid concentration was an early and characteristic features of preeclampsia. But they reported this raised serum uric acid as early change in comparison with other biological parameter changes such as blood urea and creatinine levels. They compared the serum uric acid level of preeclampsia and normal pregnancy throughout the third trimester of pregnancy (24th weeks to term). The wider time span, including cases from 24 - 34 weeks, may account for their finding of a significant rise in the

serum uric acid level of mild preeclamptic cases. According to our study, the serum uric acid levels in preeclampsia and normal pregnancy were compared in the 34-38 weeks of gestation and no significant rise is found in mild preeclamptic cases. The mean serum uric acid level in mild preeclampsia compared with normal pregnancy at the same gestational period was not significantly different. Therefore the serum uric acid level may be of little use as predicting sign of preeclampsia.

ACKNOWLEDGEMENTS

We wish to express our sincere thanks to Professor Lt. Col. Myint Maung Maung and Professor Daw Khin Nyunt for permission to conduct this research work at their departments.

REFERENCES

1. McFarlane, C.N. An evaluation of the serum uric acid level in pregnancy. J. Obstet. Gynec. Brit. Cwlt. 1963; 7: 63-67.
2. Haynes, D.M. Toxaemia of pregnancy. In: Haynes D.M. ed. Medical Complications During Pregnancy. Mc GrawHill Book Company. The Blackiston Division. 1969; 1-66.
3. Weir, R.J. Hypertension and eclampsia in pregnancy. In Donald I. ed. Practical Obstetric Problems. 5th Ed. Lloyd-Luke (Medical Books) Ltd. London. 1979; 284-320.
4. Carroll, T.J., Coburn, H., Douglas, R. & Babson, A.L. A simplified alkaline phosphotungstate assay for uric acid in serum. Clin. Chem. 1971; 17: 158-160.
5. W.H.O. Technical Report Series, No. 628, 1978 (Arterial hypertension. Report of a W.H.O Expert Committee).
6. Varley, H., Gowenlock, A. H. & Bell, M. Practical Clinical Biochemistry. William Heineman Medical Books Ltd. London. (5th Ed.) 1980; 1: 600.
7. Slemon, J.M., Bojert, L. J. The uric acid content of maternal and foetal blood. J. Biol. Chem. 1917; 32: 63. Cited by Dunlop W & J.M. Davison. The effect of normal pregnancy upon the renal handling of uric acid. Brit. J. Obstet. Gynec. 1977; 84: 13-21.
8. Seitchik, J. Observation on the tubular reabsorption of uric acid. Am. J. Obstet. Gynec. 1953; 65: 981-985.
9. Hayashi, T. Uric acid and creatinine clearance studies in normal pregnancy and toxemia of pregnancy. Am. J. Obstet. Gynec. 1956; 71: 859-874.
10. Fay, R.A., Bromham, D.R., Brooks, J.A., Chir, B. & Gebiski, V.J. Platelets and uric acid in the prediction of preeclampsia. Am. J. Obstet. Gynec. 1985; 52: 1038-1039.
11. Cadden, J.F. & Stander, H.J. Uric acid metabolism in eclampsia. Am. J. Obstet. Gynec. 1939; 37: 37-46.
12. Lancet, M. & Fisher, J. The value of blood uric acid levels in toxemia of pregnancy. J. Obstet. Gynec. Brit. Emp. 1956; 63: 116-119.
13. Pollak, V.E. & Nettles, J.B. The kidney in toxemia of pregnancy a clinical and pathological study based on renal biopsies. Medicine. 1960; 39: 469-526.
14. Redman, C.W.G., Beilin, L.J. & Bonnar, J. Renal function in pre-eclampsia. J. Clin. Path. (Suppl. Royal Colledge of Pathologists). 1976; 10: 91-94.

Accepted for publication 13 June 1990.

Vero cell cytotoxic Escherichia coli isolated from children with diarrhoea

Mar-Mar-Nyein, Kyu-Kyu-Mar, Tin-Aye & Hayashi, H.

Bacteriology Research Division
Department of Medical Research

A total of 616 isolates of Escherichia coli from 90 diarrhoea cases and 87 control cases were included in this study. Vero cells were cultured in microtitre plates. E. coli toxin was produced by growing in Trypticase Soy Broth with vigorous shaking. It was demonstrated that 17 out of 55 cases (30.9 per cent) from diarrhoea group and 7 out of 70 cases from control group (10 per cent) were verotoxic and these were not Enteropathogenic (EPEC) nor Enterotoxigenic (ETEC) group of E. coli. It was also observed that in diarrhoea group of study, 5 out of 9 cases of (EPEC) and 2 out of 8 cases of (ETEC) and 8 out of 18 cases of both (EPEC + ETEC) were verotoxic. Other bacteria tested failed to produce toxic substances which could produce cytopathic effect on Vero cells.

INTRODUCTION

Konowalchuk and coworkers(1) first reported that culture filtrates of isolated Escherichia coli produced cytotoxin differed from the heat stable or heat labile enterotoxin. It was cytotoxic for vero cells but not for Y-1 or CHO cells. It was labile to heat and antigenically different from heat labile enterotoxin and membrane filtration indicated a molecular weight of 10,000 to 30,000. It was also shown that some of the strains producing verotoxin belonged to traditional infantile enteropathogenic (EPEC) serogroups. Scotland et al.,(2) also determined the frequency of verotoxin production to be rare in EPEC and found in serogroups 018, 026, 0111, 0126 and 0128. Wade et al.,(1979) tested culture filtrates of 252 strains of E. coli obtained from 56 children with diarrhoea and reported that 3 strains were showing verotoxic and subsequently they were identified as 026. O'Brien and coworkers(4) reported that verotoxin is probably identical to shiga toxin the

cytotoxin produced by Shigella dysenteriae type 1. In North America, Johnson et al., (1983) and O'Brien et al., (1983) reported that verotoxin producing strains of E. coli serotype 0157 H7 have been shown to be the epidemic agents in outbreaks of haemorrhagic colitis and verotoxin positive strains of serogroups of 026, 0111, 0113 and 0157 have been implicated in cases of haemolytic uraemic syndrome (HUS). It remains to be shown that verotoxin has a role in the pathogenesis of diarrhoea. This study is to examine verotoxic effect on some Enteropathogenic (EPEC), Enterotoxigenic (ETEC) and E. coli which do not produce either EPEC or ETEC (untypable UT).

MATERIALS AND METHODS

Specimens:

616 strains of isolates (320 strains from diarrhoea and 296 strains from control) isolated from children under three years of age from Yangon Children Hospital were included in this

study. Thirty two strains were Enteropathogenic *E. coli* (EPEC); 18 strains were Enterotoxigenic *E. coli* (ETEC); 42 strains were both producing EPEC and ETEC, 501 strains were untypable (non EPEC and non ETEC); 33 strains were other bacteria such as *Salmonella*, *Shigella*, *Klebsiella*, *Plesiomonas*, *Edwardsiella* and *Vibrios*. These were isolated from 90 cases of diarrhoea cases and 87 cases of control cases.

Preparation of *E. coli* toxin

E. coli strains were inoculated in 1.5 ml of Trypticase Soy Broth (BBL Products) in universal container and shaken at 37°C overnight (280 rpm/min, Takasaki shaker). The culture was spun at 10,000 rpm at 4°C for 30 minutes and the supernatant stored at 4°C until cell culture assay.

Cell culture assay

Vero cells were passed by trypsinization and grown as monolayers at 37°C in a 5 per cent carbondioxide atmosphere. Minimum Eagles Medium (MEM) with glutamine supplemented with 10 per cent calf sera and 1 per cent foetal calf sera were used for growing the cells. For maintaining the cells, MEM with glutamine and 2 per cent foetal calf sera were used. Toxin activity was assayed in 96 well microtitre flat bottom plates (Falcon 3072). Three days prior to assay, vero cells were cultured in the growth media containing 10 cells approximately in each well to get a monolayer. The medium was not changed at the time of assay. To 0.2 ml of cell culture, 0.02 ml of bacterial filtrate was added without filtering through the millipore filters. For negative controls, medium only and *E. coli* ATCC 25922 were used. For positive controls, *Shigella dysenteriae* I and *E. coli* 0 128 culture filtrates were used. Then the plates were incubated at 37°C with 5 per cent carbondioxide. Titres in ten folds and two folds were made and again tested if the filtrate proved positive.

Morphological effect was recorded as 1,2,3, and 4 and these ratings corresponding to roughly 25,50,75 or 90 per cent of cells affected on day 1 to day 4. Simultaneously, the toxin were boiled in a boiling waterbath for 15 mins. and tested for heat sensitivity. In some cases antishiga toxin was administered in cell assay to see whether they could be neutralised or not. After 4 days, the cells were fixed with methanol for 10 mins. and stained with Giemsa's solution (BDH) for 30 minutes. It was then air dried and then checked again under microscope.

RESULTS

Figure 1 shows the normal Vero cells fixed firmly to the surface of the plate in monolayer form. The effect of *E. coli* supernatant on vero cells was shown in Figure 2. Microscopically, the Vero toxin affected cells appeared round, but shrivelled, many floated in the medium. Undiluted culture filtrates induced the above effect in at least 50 per cent of the monolayer within 24 hours, but the

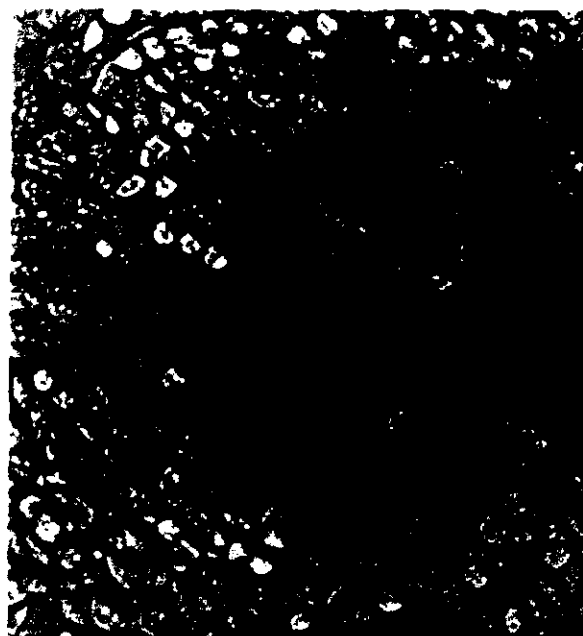


Figure 1. Normal vero cells



Figure 2. Cytotoxic vero cells

cytopathic effect advanced with time. Maximum titre limit of examination was done in day 4. The titre of the toxin varied within the strains from undiluted toxin to 1024. It was considered verotoxic only when the titre of supernatant was more than 1:64. The vero toxin response was readily distinguished from that of *E. coli* heat labile toxin (LT) in vero cells. With LT, the cells were enlarged, thick walled, refractile and possessed several filamentous tendrils (Figure 3). In some cases of LT, the effect on the cells faded with time after 2 or 3 days, the monolayers appeared normal, but the VT affected cells did not recover even after 4 days of examination.

Table 1 shows the verotoxic response of Enteropathogenic (EPEC) strains. A total of 32 strains of EPEC were tested and it was observed that 6 out of 32 strains were found to be verotoxic with undiluted culture filtrates. When tested again with 2 fold dilutions it was found that 4 strains were showing to be verotoxic with more than 1:64 dilutions.

Table 2 shows the enterotoxigenic *E. coli* (ETEC) isolated from

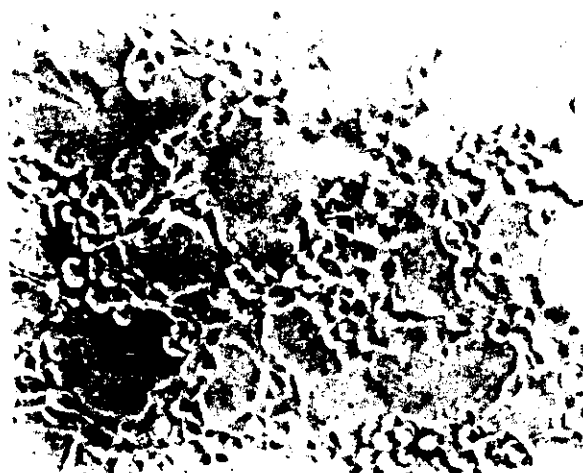


Figure 3. *E. coli* (LT) toxin treated vero cells

children with diarrhoea and control groups. It was found that 5 out of 18 strains were showing verotoxic; 2 of them were LT, 2 were STLT and 1 was ST producing *E. coli*. When tested again with two fold dilutions it was found that only 2 of them were verotoxic.

Table 3 shows the *E. coli* strains that possessed both EPEC and ETEC. It was observed that 13 out of 32 strains were verotoxic with undiluted culture supernatant. When tested with 2 fold dilutions it was observed that 6 strains were verotoxic with more than 1:64 dilutions.

Table 4 shows the strains of *E. coli* with unknown serogroups that produced cytopathic effect on vero cells. In a diarrhoea group it was observed that 20 strains out of 11 cases in group A and 6 strains from 6 cases from Group C were showing verotoxic. In the control group, it was observed that 10 strains out of 5 cases in group B and 2 strains out of 2 cases in group D were verotoxic. A total of 17 cases in diarrhoea cases and 7 cases from control were possessing verotoxic *E. coli* and this was found to be significant.

Simultaneously, other bacteria tested were shown in Table 5. In *Salmonella* group, the species included were *S.*

Table 1. Verotoxic E. coli from EPEC

< 1 y e a r								1 - 3 y e a r s							
Case No.	Lab No.	A(Diarrhoea) 12 strains	Vero	Case No.	Lab No.	B(Control) 10 strains	Vero	Case No.	Lab No.	C(Diarrhoea) 6 strains	Vero	Case No.	Lab No.	D(Control) 4 strains	Vero
17	201	O 26 H 36	+	6	81	O 114 H 32	-	14	167	O 125 H 27	+	9	170	O 146 H 19	-
	203	O 26 H 3	-	25	427	O 126 H 31	-	16	236	O 125 H -	+		171	O 146 H 19	-
*19	218	O 25 H 12	-	40	493	O 125 H 12	-		238	O 125 H -	-	16	257	O 55 H 27	-
	220	O 25 H 12	-		494	O 127 H 1	-		239	O 125 H -			259	O 55 H 27	-
21	223	O 86 H 21	+	47	595	O 125 H 12	-	*20	333	O 124 H -	-				
	224	O 86 H 21	-		596	O 125 H 12	-		334	O 112 H -	-				
15	178	O 114 H 2	-	48	667	O 111 H 45	-								
	179	O 114 H 2	-	*53	645	O 111 H 4	-								
	181	O 114 H 2	-	56	677	O 44 H 18	-								
32	409	O 119 H 6	+		678	O 125 H 21	-								
	431	O 119 H 6	-												
54	620	O 125 H 12	-												

*These cases are also found in ETEC or EPEC & ETEC groups

Table 2. Verotoxic E. coli from ETEC

< 1 y e a r								1 - 3 y e a r s							
Case No.	Lab No.	A(Diarrhoea) 9 strains	Vero	Case No.	Lab No.	B(Control) 5 strains	Vero	Case No.	Lab No.	C(Diarrhoea) 2 strains	Vero	Case No.	Lab No.	D(Control) 2 strains	Vero
16	198	LT OUT H 7	+	7	297	LT OUT H 41	-	*12	114	LT OUT HBT	-	28	651	STLT OUT H-	+
*19	217	STLT OUT H 10	-	13	141	LT OUT H 13	-	*14	168	STLT OUT HBT	-		652	STLT OUT H-	+
31	385	LT OUT H -	-	17	209	ST OUT H -	-								
	387	LT OUT H -		29	470	LT OUT H 41	-								
39	464	LT OUT H 12	-	*53	642	ST OUT HBT									
	465	LT OUT H 12	-												
	466	LT OUT H 12	-												
43	501	LT OUT H 9	+												
48	564	ST OUT H 16	-												

* These cases are also found in EPEC or EPEC & ETEC

Table 3. Verotoxic E. coli from EPEC & ETEC

< 1 y e a r								1 - 3 y e a r s							
Case No.	Lab No.	A(Diarrhoea) 13 strains	Vero	Case No.	Lab No.	B(Control) 1 strain	Vero	Case No.	Lab No.	C(Diarrhoea) 17 strains	Vero	Case No.	Lab No.	D(Control) 1 strain	Vero
5	10	ST O 128 H27	+	45	660	ST O 86 H18	+	1	3	ST O 126 H12	-	41	148	LT O 8 H11	+
	27	ST O 128 H27	+					2	7	ST O 8 H 9	+				
10	77	LT O 123 H -	-					5	38	ST O 126 H12	-				
11	87	ST O 126 H12	-					7	47	STLT 0126H2	+				
20	229	ST O 27 H20	+					8	98	STLT 0159H16	+				
	232	ST O 153 H10	-						99	STLT 0159H16	+				
26	298	LT O 58 HUT	-						100	STLT 0159H16	+				
	299	LT O 58 HUT	-					*12	117	ST 0126HUT	-				
	300	LT O 58 HUT	-					13	155	LT O 25H-	+				
30	376	ST O 25 H-	+					*14	164	STLT 0159H16	+				
44	513	ST O 78 H-	-						166	STLT 0159H16	-				
	514	ST O 78 H-	-					*20	440	ST O 8 H 9	-				
50	568	ST O 86 H11	-						441	ST O 8 H 9	-				
									442	ST O 8 H 9	-				
								25	517	LT O 159 H34	-				
									518	LT O 159 H34	-				
									519	LT O 25 H -	-				

* These cases are also found in EPEC or ETEC

Table 4. Titre of E. coli toxin showing cytopathic effect on vero cells
(unknown serogroup)

< 1 year						1 - 3 years					
A (DIARRHOEA) 20 Strains			B (CONTROL) 10 Strains			C (DIARRHOEA) 6 Strains			D (CONTROL) 2 Strains		
Case No.	Lab No.	Titre	Case No.	Lab No.	Titre	Case No.	Lab No.	Titre	Case No.	Lab No.	Titre
18	60	1:1024	66	250	1:32	29	91	1:1024	48	172	1:32
32	101	1:128		251	1:64	53	192	C	67	253	1:1024
	105	1:64		252	1:64	67	253	1:32			
	106	C.	108	414	1:1024	101	407	N.T.			
58	212	1:64	145	573	1:256	88	358	1:64			
	213	1:8	157	623	1:1024	133	522	1:256			
65	249	1:1025		624	1:1024						
76	266	1:256	172	668	1:1024						
	269	1:1024		669	1:256						
	267	1:64		670	1:256						
74	276	1:256									
38	351	1:128									
92	343	1:1024									
	373	1:512									
	374	1:128									
94	378	1:64									
	379	1:64									
	380	1:1024									
103	412	1:1024									
152	608	1:256									

C. = Undiluted toxin,

N.T. = Not tested for double dilution

Table 5. Other bacteria tested for vero cell cytotoxicity

	< 1 year		1 - 3 years		Total
	A (Dia.)	B (Cont.)	C (Dia.)	D (Count.)	
<u>Klebsiella</u>			3	1	4
<u>Plesiomonas</u>		3	2	3	8
<u>Edwardsiella</u>		1	1		2
<u>Shigella sonnei</u>			1	1	2
<u>Salmonella</u>	1	8		2	11
<u>V. cholerae</u>			4		4
<u>Vibrios (others)</u>			1	1	2
Total	1	12	12	8	33

derby, S. enteritidis, S. krefeld, S. typhimurium, S. senftenberg, S. stanley and S. paratyphi B. It was observed that only S. enteritidis and S. stanley species showed verotoxic with very low titre (i.e. 1:4 and 1:8). In V. cholerae Inaba (CT) it also showed verotoxic but the cells were not dead at the titre of 1:2. Thus other bacteria tested were not producing toxic substances which could produce cytopathic effect on vero cells.

To summarize the findings, it was shown in Table 6 that 32.2 per cent of less than 1 year age group and 41.9 per cent of 1 to 3 years age group of children with diarrhoea were having E. coli that could produce cytopathic effect on Vero cells. In a control group of the study 11.9 per cent from less than 1 year age group and 17.9 per cent from 1 to 3 years age group were possessing Vero toxic E. coli. In total, 35.6 per cent from diarrhoea and 13.8 per cent from control were possessing E. coli which produced cytopathic effect on Vero cells. It was found to be significant between the two groups studied.

DISCUSSION

E. coli is one of the several agents that cause intestinal disease in humans and animals. Four classes of E. coli have been recognised. They are Enterotoxigenic E. coli (ETEC) strains that produced heat labile (LT) enterotoxin and/or heat stable (ST) enterotoxin. Enteroinvasive E. coli (EIEC) strains like Shigella, penetrate and multiply within epithelial cells. Enteropathogenic E. coli (EPEC) strains belong to certain serogroups that have been incriminated as pathogens by epidemiological studies. Some EPEC strains have been shown to adhere to cells of the intestinal mucosa and to produce pathognomonic lesion at the site of attachment. Enterohaemorrhagic E. coli (EHEC) strains cause clinical syndrome (haemorrhagic colitis) and serotype O157 H7 has been shown to be associated with this illness. Neither EPEC nor ETEC strains produced classic enterotoxins nor are they enteroinvasive. Some strains of E. coli produce a cell associated cytotoxin that is neutralised by antibodies against purified shiga toxin from Shigella dysenteriae 1. The cytotoxin purified from one EPEC strain 026 H11 (H30) and from one EHEC

Table 6. Distribution of verotoxic E. coli in children

Types	< 1 year		1 - 3 years		< 1 & 1-3 years	
	A (diarrhoea)	B (Control)	C (Diarrhoea)	D (Control)	Diarrhoea	Control
UT	11/39 (28.2)	5/46 (10.9)	6/16 (37.5)	2/24 (8.3)	17/55 (30.9)	7/70 (10)
EPEC	3/6 (50)	0/7 (0)	2/3 (66.7)	1/2 (50)	5/9 (55.6)	1/9 (11.1)
ETEC	2/6 (33.3)	1/5 (20)	0/2 (0)	1/1 (100)	2/8 (25)	2/6 (33.3)
EPEC + ETEC	3/8 (37.5)	1/1 (100)	5/10 (50)	1/1 (100)	8/18 (44.4)	2/2 (100)
Total <u>E. coli</u>	19/59 (32.2)	7/59 (11.9)	13/31 (41.9)	5/28 (17.9)	32/90 (35.6)	12/87 (13.8)
Other bacteria	0/1 (0)	2/12 (16.7)	1/12 (8.3)	0/8 (0)	1/13 (7.7)	2/20 (10)

Figures in parenthesis denote percentage

strain 0157 H7(933) which cause cytotoxicity to Hela and Vero cells; lethality for mice and enterotoxigenicity for ligated ileal segment from rabbits showed subunit structure similar to those of shiga toxin as reported by O'Brien et al in 1983 and 1984 (4,7,8). Verotoxin (shiga - like) associated with haemorrhagic colitis or haemolytic uraemic syndrome was shown by various workers such as Gransden et al., 1985 (9); Karmali et al., 1985; 1986 (10,11); Scotland 1985 (12); Rose et al., 1985 (13). For experiments reported in their studies there is evidence that 2 cytotoxins active against Vero cells were reported by 0157 strains.

It was reported by Marques 1986 (14) that the levels of cytotoxin produced by *E. coli* strains varied from low to high levels and reported that all strains of *E. coli* tested by them made at least small amounts of cell associated cytotoxin (since some strains with no detectable activity in sonic lysates showed cytotoxic in lysates prepared by French pressure method). However, low level cytotoxicity of some strains were not neutralised by antibody to shiga toxin. Cell associated cytotoxicity not neutralised by antibody to shiga toxin had been reported by O'Brien et al., 1982 (15) and Cleary et al., 1985 (16). Konowalchuk et al., 1977 (1) reported that the extracellular diarrhoea with serogroup O 128 and one strain isolated from suckling pig with diarrhoea whose serogroup is 0138 was antigenically different from shiga-like-toxin. Smith et al., in 1983 (17) also detected antigenic variation of extracellular cytotoxin from strain of *E. coli* 0157.

As shown in Table 6, it was found that verotoxic *E. coli* possessing with EPEC alone or possessed with EPEC and ETEC showed high incidence than ETEC alone. It might be the fact that EPEC virulence plays an important role. It was also observed that 30.9 per cent of untypable *E. coli* showed verotoxic. These untypable cases were not

associated with other pathogens. Thus it seemed that these *E. coli* might play an important role in causing diarrhoea in children. More research is needed to identify the toxin as well. Other bacteria besides *E. coli* were found to be of very low titre and very few organisms showed verotoxic.

These findings suggest that cytotoxins produced at elevated levels play a role in pathogenesis of acute diarrhoea. It was also found that strains isolated from humans without illness made cytotoxin at much lower levels than did some strains from sources of diarrhoea. Cytotoxin may be only a virulence determined for *E. coli* when it is produced in large amounts. Small amount of cytotoxin, however could damage test cells if delivered by strains of *E. coli* that adhere vividly to or that invade intestinal epithelial cells. However, it should be emphasised that the amount of cytotoxin produced in vitro may not correlate with the amount produced in vivo.

ACKNOWLEDGEMENTS

We would like to express our gratitude to Dr. Aung - Than - BaTu, former Director General and Dr. May-May-Yi, Director-General of Department of Medical Research for their keen interests and valuable advice in conducting this research.

Also to Dr. Than-Swe, Deputy Director DMR for invaluable advice and necessary helps given. To Dr. Thane-Toe and Dr. Mi-Mi-Khin for their keen interests and encouragements. This research was supported by Grant from Japan International Cooperation Agency under the Project title of Research on Treatment of infectious Diseases of the alimentary system.

REFERENCES

1. Konowalchuk, J., Spiers, J.I., & Stavric, S. Vero response to a

- cytotoxin of Escherichia coli. Infect. Immun. 1977; 18: 775-779.
2. Scotland, S.M., Day, N.P. & Rowe, B. Production of a cytotoxin affecting Vero cells by strains of Escherichia coli belonging to traditional enteropathogenic serogroups. F.E.M.S. Microbiol. Let. 1980; 7: 15-17.
3. Wade, W.G., Thom, B.T. & Evan, N. Cytotoxic enteropathogenic Escherichia coli. Lancet 1979; 2: 1235-1236.
4. O'Brien, A.D., Lively, T.A., Chang, T.W. & Gorbach, S.L. Purification of Shigella dysenteriae 1 (Shiga)-like toxin from Escherichia coli 0157:H7 strain associated with haemorrhagic colitis. Lancet 1983; ii: 573.
5. Johnson, W.M., Lior, H., Bezanson, G.S. Cytotoxic Escherichia coli 0157:H7 associated with haemorrhagic colitis in Canada. Lancet 1983; i: 76.
6. O'Brien, A.D., Lively, T.A., Chen, M.E., Rothman, S.W. & Formal, S.B. Escherichia coli 0157:H7 strains associated with haemorrhagic colitis in the United States produce a Shigella dysenteriae 1 (Shiga)-like toxin. Lancet 1983; i: 702.
7. O'Brien, A.D. & Laveck, G.D. Purification and characterization of Shigella dysenteriae 1-like toxin produced by Escherichia coli. Infect. Immun. 1983; 40: 675-683.
8. O'Brien, A.D., Newland, J.W., Miller, S.F., Smith, H.W., Formal, S.B. & Holmes R.K. Shiga-like toxin. Converting phages from Escherichia coli strains that cause haemorrhagic colitis or infantile diarrhoea. Science 1984; 226: 694-696.
9. Gransden, W.R., Damm, S. & Anderson, J.D. Verocytotoxin producing Escherichia coli 0157:H7 and diarrhoea. Annals. Int. Med. 1985; 103: 160.
10. Karmali, M.A., Petric, M., Lim, C., Fleming, P.C., Arbus, G.S. & Lior, H. The association between idiopathic haemolytic uraemic syndrome and infection by verotoxin producing Escherichia coli. J. Infect. Dis. 1985; 151: 775-782.
11. Karmali, M.A., Petric, M., Louie, S. & Chung, R. Antigenic heterogeneity of Escherichia coli verotoxins. Lancet 1986; i: 164-165.
12. Scotland, S.M., Smith, H.R. & Rowe, B. Two distinct toxins active on vero cells from Escherichia coli 0157. Lancet 1985; ii: 885-886.
13. Rose, P.E. Armour, J.A., Williams, C.E. & Hill, F.G.H. Verotoxin and neuraminidase induced platelet aggregating activity in plasma: their possible role in the pathogenesis of the haemolytic uraemic syndrome. J. Clin. Pathol. 1985; 38: 438-441.
14. Merques, L.R., Moore, M.A., Willis, J.G., Wachsmuth, I.K. & O'Brien, A.D. Production of Shiga-like toxin by Escherichia coli. J. Infect. Dis. 1986; 154: 338-341.
15. O'Brien, A.D. & Laveck G.D. Immunological and cytotoxic activity of Shigella dysenteriae 1 (shiga) and shiga-like toxin. Infect. Immun. 1982; 35:1151-1154.
16. Cleary, T.G., Mathewson, J., Faris, E. & Pickering L.K. Shiga-like cytotoxin production by Enteropathogenic Escherichia coli serogroups. Infect. Immun. 1985; 47: 335-337.
17. Smith, W.H., Green, P. & Parsell, Z. Vero cell toxin in Escherichia coli and related

bacteria transfer by phage and
conjugation and toxic action in
laboratory animals, chickens and

pigs. J. Gen. Microbiol. 1983; 129:
3121-3137.
Accepted for publication 13 June 1990.

Detection of Escherichia coli enterotoxin from stool samples directly by Bead ELISA method

Mar-Mar-Nyein*, Oku Y.**, Than-Saw*, Tin-Aye* & Takeda T.**

*Bacteriology Research Division, Department of Medical Research

**Department of Bacterial Infection, University of Tokyo

Bacterial protein toxins as Escherichia coli enterotoxigenic heat labile toxin (LT), E. coli shigella like toxin I (VT1), E. coli shigella like toxin II (VT2) and cholera toxin were detected directly from stool using Bead ELISA method. A total of 390 stools from adults with diarrhoea and dysentery from 2 Military Hospital, Workers Hospital, Yangon General Hospital and Infectious Diseases Hospital were included in this study. It was observed that 9, 5 and 10 samples of stools showed to possess LT, VT1, VT2 toxin respectively. Some of the samples showed to possess either 2 or 3 toxins in combination. Of these, 2 samples showed to possess LT and VT1 toxins; 3 samples showed to possess LT and VT2 toxins; 11 samples showed to possess VT1 and VT2 toxins and 14 samples showed to possess LT, VT1 and VT2 toxins. Thus a total of 54 samples (13.8 per cent) showed to possess bacterial toxins. It was also observed that 28 samples out of 199 cases (14.1 per cent); 20 out of 109 cases (18.3 per cent) and 6 out of 82 cases (7.3 per cent) showed to possess bacterial toxins in January, June and November 1988 respectively. Moreover, cholera toxin was detected from 5 out of 25 cases tested.

INTRODUCTION

Several immunological methods have been established to detect various enterotoxins, such as Passive Immune Haemolysis (PIH), a Reversed Passive Haemagglutination test (RPH), a modified ELEK test (BIKEN), GM1 ELISA and Radioimmunoassay. Among these assays Enzyme Linked Immunosorbent Assay (ELISA) is the most widely used because it is rapid, accurate, simple and highly sensitive. Thus, ELISA has been applied for detection of various enterotoxins such as heat labile Escherichia coli toxin (LT), Shiga toxin (VT1) and Shigella like toxin II of E. coli (VT2) and cholera toxin (CT). To detect enterotoxin in stool it is the most convenient method for the diagnosis of diarrhoea diseases. Thus,

we attempted to do this by applying Bead ELISA method of Oku et al. (1) directly in testing stool samples to detect heat labile enterotoxin, shiga like toxin VT1 and VT2 of E. coli.

MATERIALS AND METHODS

390 stools from patients with diarrhoea and dysentery from Infectious Diseases Hospital, 2 Military Hospital, Yangon General Hospital and Workers Hospital were examined. Stools and the enrichment cultured stools were examined by Bead ELISA. For direct stool samples, 3 dilutions were made by mixing with Buffer A. The constituents were 10 mM sodium phosphate buffer, 0.1 M sodium

chloride, 0.1 per cent sodium azide and 0.1 per cent Bovine serum Albumin, and pH 7.00. Dilutions of 1/5, 1/25 and 1/125 were made. Then the polystyrene beads coated with antitoxin IgG were placed in tubes and incubated at 4°C overnight.

For enrichment culture stools, one loopful of stool samples were incubated with 3 ml of Trypticase Soy Broth at 37°C overnight in a shaking waterbath. Polymixin B was added and again incubated for 1 hour. It was then spun at 10,000 rpm for 10 minutes. The supernatant was mixed with Buffer A for half strength and polystyrene beads coated with antitoxin IgG was placed in tubes and incubated at 37°C for 1 hour.

The above polystyrene beads were washed twice with 0.01 M sodium phosphate buffer pH 7.0 containing 0.1 M sodium chloride (Buffer B). The bead was then incubated at 37°C for 1 hour with Fab-Horse Radish peroxidase

conjugate (200 ng/ml) in Buffer B containing 2 per cent bovine serum albumin. The polystyrene bead was washed twice with Buffer B and transferred to a new test tube. Peroxidase activity bound to the polystyrene bead was then assayed by adding 0.56 mM 3,3', 5,5' - tetramethylbenzidine (TMBZ) in 0.1 M sodium acetate buffer (pH 5.5) as a substrate and incubated at 30°C for 5 minutes. Then 0.02 per cent hydrogen peroxide was added and incubated at 30°C for additional one hour. The reaction was stopped by adding 0.2 ml of 4N sulphuric acid and the resulting yellow colour was measured at 450 nm in a spectrophotometer (Oku et al., 1988).

RESULTS

Table 1 shows a direct detection of LT, VT1 and VT2 from stool samples. It was categorized tentatively into seven groups. In Group 1, 14 samples showed

Table 1. Detection of *E. coli* heat labile toxin (LT); shigella-like toxin I (VT1); *E. coli* shigella-like toxin II (VT2) from patients in January, June and November 1988 (Direct from stool).

group	LT	VT1	VT2	Jan.	Jun.	Nov.	Total
				109	109	82	390
I	+	+	+	9	5	0	14(3.59)
II	+	+	-	0	2	0	2(0.51)
III	-	+	+	6	4	1	11(2.82)
IV	+	-	+	3	0	0	3(0.77)
V	+	-	-	3	2	4	9(2.31)
VI	-	+	-	2	2	1	5(2.31)
VII	-	-	+	5	5	0	10(2.56)
Total				28(14.4)	20(18.3)	6(7.3)	54(13.8)
VIII	-	-	-	171	89	76	336

Figures in parenthesis denote percentages

positive for all toxins (3.59 per cent). Group II was categorized as LT and VT1 positives and 2 cases were found to be positive (0.51 per cent). Group III was categorized as VT1 and VT2 positives and in this group 11 cases proved positive (2.82 per cent). In Group IV, LT and VT2 positives were found in 3 cases (0.77 per cent) while in Group V, LT alone was detected in 9 cases (2.31 per cent). In Group VI, only VT1 positive was found in 5 cases (2.31 per cent). In the last Group VII, only VT2 positive was found in 10 cases (2.56 per cent). Totalling all the cases, 54 samples were found to contain bacterial protein toxins (13.8 per cent). It was also observed that 14.1, 18.3 and 7.3 per cent were positive to possess bacterial protein toxins by BEAD ELISA in January, June and November respectively.

Table 2. Effect of enrichment incubation of stool samples on the detection of LT, VT1, & VT2

Toxin detected	Direct	Incubation
	281	281
LT	7	11
VT1	3	5
VT2	5	8
LT+VT1	0	3
LT+VT2	3	2
VT1+VT2	7	3
LT+VT1+VT2	9	9
Total	34(12.1)	41(14.6)

Figures in parenthesis denote percentages

Table 2 shows the effect of enrichment incubation of stool samples on the detection of LT, VT1 and VT2. A total of 281 cases were compared. It was found that incubation in enriched media raised the detection ratio from 12.1 per cent to 14.6 per cent. LT production apparently accelerated during the incubation because LT (-) samples became LT (+) in eight samples, while LT (+) samples became LT (-) in 2 samples for reasons unknown.

VT1 or VT2 production seems to be more delicate because it is affected by Fe^{++} concentration. There appears to be some degradative effect on produced toxin during incubation by protease or other digestive enzymes. But as far as LT is concerned the effect seemed less due to incubation.

Table 3 shows the presence of cholera toxin direct from stools and from enrichment cultures. It was noted that 5 samples were showing to possess cholera toxin direct from stools and from enrichment cultures out of 25 cases tested (20.0 per cent).

Table 3. Detection of cholera toxin
direct from stool

No. cases tested	No. cases detected	No. cases detected
	Direct	Enrichment
25	5(20)	5(20)

Figures in parenthesis denote percentages

DISCUSSION

Tin Aye et al. (2) reported that bacterial pathogens which are responsible for diarrhoea includes, *Escherichia coli* (heat labile and heat stable toxins) 22.7 per cent; *Shigella* (1.8 per cent); *Salmonella* (0.3 per cent).

Two mechanisms have been described by which *E. coli* can cause diarrhoea disease in humans and animals by Dupont et al. (3). Some strains colonize in the small bowel and cause fluid secretion by elaboration of heat labile (LT) and/or heat stable (ST) enterotoxins (3,4,5,6). In contrast to these noninvasive enterotoxic organisms, Ogawa et al. (7) reported that certain strains of *E. coli* penetrate and multiply within colonic epithelial cells and are able to produce keratoconjunctivitis in guinea pigs named as the Sereny Test by Sereny (8). Infection with these enteroinvasive strains may cause symptoms that mimic those seen in patients with shigellosis was demonstrated by Sakasaki et al. (9). That *E. coli* must be capable of mediating gastroenteritis by other means is indicated by the finding that some strains associated with diarrhoeal disease produce no detectable LT or ST and are Sereny test negative (Cantey & Blake 10; Levine et al. 11; Ushen & Rolla, 12). Instead, such organisms have been shown by electron microscopic studies to displace the microvilli and adhere to the cell membrane of intestinal epithelial cells but apparently with less frequency and perhaps by a mechanism different from that of shigellae (Takeuchi et al. 13).

O'Brien et al. (14,15) indicated that some of this LT negative, ST negative, Sereny test negative organisms can produce a toxin like that of *Shigella dysenteriae* type I (shiga) and suggested that this toxin could be responsible for the diarrhoea-evoking potential of these microbes. Thus *E. coli* which produce like shigella toxin VT1 and VT2 may also play an important role in diarrhoeal

and dysentery diseases.

Oku et al. (1) reported that the sensitivity of BEAD ELISA was found to be quite high with various bacterial toxins. The sensitivity was less than 20 pg/ml for thermostable direct haemolysin of *Vibrio parahaemolyticus*; less than 60 pg/ml for shiga toxin (VT1); less than 20 pg/ml for VT2 (shiga like II of *E. coli*); less than 200 µg/ml of heat labile enterotoxin of *E. coli* and less than 6 µg/ml of cholera enterotoxin.

From our study, it was found that 13.8 per cent of samples were found to contain either one or two or all three toxins tested. Some of the samples were neutralized by its respective antitoxins and found to be specific. Detection of two or more toxins indicated that the patients might have a mixed infection.

It was found that BEAD ELISA was sensitive in detection of bacterial toxins from stool samples and could be detected within 48 hours, whereas by routine diagnosis it will take more than 72 hours. From our findings it was shown that enterotoxin could be detected directly from fresh stool samples. The detective ratio was 12.1 per cent in cool dry and 18.3 per cent in hot wet seasons, which was well correlated with the isolation ratio of enteropathogenic and enterotoxigenic *E. coli*. VT2 toxin was detected in a higher ratio than expected. It should also be noticed that vero cell cytotoxic toxins were distributed in high ratio in these cases. In conclusion, complex infection of *E. coli* that produced each of different toxin or infection of *E. coli* that produced more than two different toxins are suspected in many cases.

ACKNOWLEDGEMENTS

We would like to thank Japan International Cooperation Agency (JICA) for

providing all the materials required for this study. We express our gratitude to our former Director General of Department of Medical Research Dr. Khin Maung Tin and Director General Dr. May May Yi and Director Dr. Than Swe for their constant interest and encouragements. Lastly to all the staff of Bacteriology Research Division for helping in implementing the project.

REFERENCES

1. Oku, Y., Uesaka Y., Hirayama, T. & Takeda, Y. Development of a highly sensitive BEAD-ELISA to detect bacterial protein toxins. Microbiol. Immunol. 1988; 32: 807-816.
2. Tin-Aye, Mar-Mar-Nyein, Kyi-Kyi-Khin, Mi-Mi-Khin, Khin-Maung-U, Myo-Khin, Aung-Myo-Han, Thane-Toe & Hayashi, H. Epidemiology and aetiology of acute childhood diarrhoea in Burma: a rural community survey. Trans. Roy. Soc. Trop. Med. Hyg. 1989; 83: 827-830.
3. Dupont, H.L., Formal, S.B., Hornick, R.B., Snyder, M.J., Libonati, J.P., Sheahan, D.G. & Labrec, E.H. Pathogenesis of Escherichia coli diarrhoea. N. Engl. J. Med. 1971; 285: 1-9.
4. Gyles, C.L. Heat-labile and heat stable forms of the enterotoxin from E. coli strains enteropathogenic for pigs. Ann. N.Y. Acad. Sci. 1971; 178: 314-322.
5. Gyles, C.L. & Barnum, D.A. A Heat-labile enterotoxins from strains of Escherichia coli enteropathogenic for pigs. J. Infect. Dis. 1969; 120: 491-426.
6. Smith, H.W. & Gyles, C.L. The relationship between the apparently different enterotoxins produced by the enteropathogenic strains of Escherichia coli of porcine origin. J. Med Microbiol. 1970; 3: 387-401.
7. Ogawa, H., Nakamura, A. & Sakasaki, R. Pathogenic properties of "enteropathogenic" Escherichia coli from diarrhoeal children and adult. Jap. J. Med. Sci. Biol. 1968; 21: 333-349.
8. Sereny, B. Experimental shigella keratoconjunctivitis: a preliminary report. Acta Microbiol. Acad. Sci. Hung. 1955; 2: 293-296.
9. Sakasaki, R., Tamura, K. & Saito, M. Enteropathogenic Escherichia coli associated with diarrhoea in children and adults. Jap. J. Med. Sci. Biol. 1967; 20: 387-399.
10. Cantey, J.R. & Blake, R.K. Diarrhoea due to Escherichia coli in the rabbit: a novel mechanism. J. Infect. Dis. 1977; 135: 454-462.
11. Levine, M.M., Berquist, E.J., Nalin, D.R., Waterman, D.H., Hornick, R.B., Young, C.R., Sotman, S. & Rowe, B. Escherichia coli strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. Lancet. 1978; 1: 1119-1122.
12. Ulshen, M.H. & Rolla, J.L. Pathogenesis of Escherichia coli gastroenteritis in man-another mechanism. N. Engl. J. Med. 1980; 302: 99-101.
13. Takeuchi, A. Inman, L.R., O'Hatley, P.D., Cantey, J.R. & Lushbaugh, W.B. Scanning and transmission electromicroscopic study of Escherichia coli 015 (RDEC-1) enteric infection in rabbits. Infect. Immun. 1978; 19: 685-694.
14. O'Brien, A.D. & Laveck, G.D. Immunological and cytotoxic activities of Shigella dysenteriae 1 (Shiga) and Shiga-like toxins. Infect. Immun. 1982; 35: 1151-1154.
15. O'Brien, A.D., Laveck, G.D., Thompson, M.R. & Formal, S.B. Production of Shigella dysenteriae type 1 - like cytotoxin by Escherichia coli. J. Infect. Dis. 1982; 146: 763-765.

Accepted for publication 13 June 1990.

The effect of chemical agents on contaminated water

Khin-Nwe-Oo, Zin-Zin-Moe*, Aung-Myo-Han** & Tin-Aye**

Bacteriology* & Epidemiology** Research Divisions
Department of Medical Research

The study was done to determine the effect of bleaching powder, alum and potassium permanganate on contaminated water. The natural water samples were collected in Yangon from February 1989 to January 1990. They were treated with these 3 chemical agents and faecal coliform(FC) count was determined by multiple tube method. Bleaching powder and alum could reduce FC by 3 logs and 2 logs within 0.5 and 1 hour respectively. However, potassium permanganate could reduce only 1 log within 1 hour. As expected, the data showed that watertreatment with chlorination was the most effective means of providing good microbiological quality of drinking water. After bleaching powder, potash alum was also found to be quite effective in cleaning the pathogens and turbidity from the water.

INTRODUCTION

Bleaching powder is standard chemical agent used in the treatment of water in waterplant. The oxidizing agent potassium permanganate (KMnO_4) has a marked bactericidal activity and was used in purifying water during the London cholera epidemic of 1866(1). Alum potash, known as "Kyauk-Chin" in Myanmar, has been used since ancient times for removing turbidity from water for domestic use. Alum acts to clear water of suspended material by flocculation and reduction of the pH(2). This study was done to determine the bactericidal effect of these 3 chemical agents on faecal coliforms presented in natural water.

MATERIALS AND METHODS

Collection of samples

The study was conducted in Yangon from February 1989 to January 1990. The natural water samples were collected from (a) taps from tube wells

in Hlaing township used for drinking purpose, (b) shallow wells in Hlaing township used for drinking purpose, (c) ponds in Dagon township not used for drinking purpose and (d) rivers in Yangon which are also not used for drinking. The samples were collected in the mornings in sterile containers by standard methods of collection(3) and were transported to the laboratory without ice and processed within one hour after on arrival. Twenty water samples were tested in each group.

Decontamination of natural waters

Concentration of alum 0.05%, bleaching powder 1 ppm and KMnO_4 1:50,000 were added to all types of natural waters. Faecal coliform(FC) count was determined after 0, 0.5, 1, 2 and 4 hours by multiple tube method as described by ICMSF method (4).

RESULTS

Bleaching powder 1 ppm caused the water to be odorous without forming

sedimentation. After adding 0.05% concentration of alum, the water turned slightly sour but there was no odour or colour changes and flocculates were formed within 1 hour. After 2 hours, sediments could be seen at the bottom of the samples and the sedimentation process was completed within 2-4 hours. The addition of KMnO_4 1:50,000 caused the water to have a fishy odour, turn pink and lose its palatability. Although flocculates were formed after 2 hours, the water did not become clear within 24 hours.

The pH of tap water was altered from 5.99 to 5.75 with bleaching powder, to 4.97 with alum and to 5.34 with KMnO_4 .

Survival of faecal coliforms in various samples tested is shown in Table 1. Bleaching powder and alum could

reduce 3 and 2 logs of FC within 0.5 and 1 hour respectively. The 3 logs reduction by the latter occurred only after 2 hours. However, potassium permanganate could reduce only 1 log within 1 hour and reduction by 3 logs was not achieved up till 4 hours.

DISCUSSION

Water can be treated before use by boiling, chlorination as chemical flocculation and sedimentation. Boiling of drinking water is possible but this could result in an increase in the household budget especially for those families with low income(5).

The data show that water treatment with chlorination was the most effective means of providing good microbiological quality of drinking water. Since

Table 1. Survival of faecal coliforms (*MPN/100 ml) in tap, well, pond and river waters containing different chemical agents

Water type	Time (hours)	Chemical agents		
		Alum (0.05%)	Bleaching powder (1 ppm)	KMnO_4 (1:50,000)
Tap	0	13	15	10
	0.5	9	0	9
	1	4	0	5
	2	0	0	0
	4	0	0	0
Well	0	0.2×10^3	0.5×10^3	0.7×10^3
	0.5	0.9×10^2	0	0.1×10^3
	1	0.2×10^2	0	0.1×10^2
	2	0	0	15
	4	0	0	3
Pond	0	0.5×10^3	0.7×10^3	0.4×10^3
	0.5	0.8×10^2	0	0.9×10^2
	1	0.2×10^2	0	0.3×10^2
	2	0	0	0.3×10^2
	4	0	0	0.2×10^2
River	0	0.1×10^4	0.5×10^4	0.6×10^4
	0.5	0.4×10^3	0	0.8×10^3
	1	0.2×10^2	0	0.4×10^3
	2	0	0	0.3×10^2
	4	0	0	0.1×10^2

*Median of 20 samples result, MPN = most probable number

it has no sedimentation effect, it will not be able to clear turbid water.

The effectiveness of potassium permanganate in purifying water was the lowest among these 3 chemical agents. It produces a fishy odour, turns the water pink and changes its palatibility and it is highly likely that it will not be acceptable by the users. Furthermore, its sedimentation process is also rather poor.

On the other hand, potash alum was found to be quite effective in cleaning the pathogens and turbidity from the water. Alum application and its sedimentation effect on turbid water has been used in Myanmar since ancient times. Most of the people in Myanmar know and accept this method of treatment. By using this 0.05% concentration of alum, water had only slight sour taste and it could be acceptable by the users. It has sedimentation effect, it removes the turbidity and it can clear the turbid water within 4 hours.

ACKNOWLEDGEMENT

This study was supported in part by the Japan International Cooperation Agency.

REFERENCES

1. Hankin, G.H. A simple method of checking cholera in Indian villages. British Medical Journal. 1989; 1: 205.
2. Khan, M.U. Preventing the transmission of cholera using alum potash. Annual Report ICDDR B. 1985; 16.
3. Cruickshank, R. Examination of water, milk and food. 11th ed. (revised reprint) Medical Microbiology. 1970; 962.
4. International Commission on Microbiological Specifications for Foods (ICMSF) Microorganisms in foods: their significance and methods of enumeration. Toronto: University of Toronto Press. 1968; 75-78.
5. Gillman, R.H. & Sillicom, P. Boiling of drinking water: can a fuel scarce community afford it? Bulletin WHO. 1985; 63: 157-163.

Accepted for publication 13 June 1990.

Effect of alum on bacteria in water

Khin-Nwe-Oo*, Aung-Myo-Han** & Tin-Aye*

Bacteriology* & Epidemiology** Research Divisions
Department of Medical Research

A disinfecting property of alum on bacteria in different water types was studied. Alum concentrations of 0.025%, 0.05% and 0.1% were added to natural waters (shallow well, pond and tube well) with and without seeded pathogens (Vibrio cholerae 01, Shigella dysenteriae type 1, enterotoxigenic Escherichia coli and Salmonella typhi). Total bacterial and faecal coliform counts as well as counts for the 4 specific pathogens were made at 0, 1, 2 and 4 hours after adding alum. The effects of different concentrations of alum on bacteria were compared. Because of the effect of alum (0.1%) was not so effective on S. typhi, we found that the best decontamination power of alum which is suitable for human was at 0.05%. It is most effective against V. cholerae and S. dysenteriae 1, removing them within 1-2 hours. Potash alum is probably a potentially useful cost-effective intervention measure for decontaminating household water in future community interventions.

INTRODUCTION

Although it is safe to drink boiled water, there are some disadvantages in using it as drinking water: extra money is spent on fuel and additional time is needed for boiling and cooling it, before use.

Chlorine tablets may be used for effectively disinfecting drinking water especially during diarrhoeal disease outbreaks. However, it is not readily available and also it gives a peculiar taste to water, so that it is not popular.

As such, other means of disinfecting drinking water are needed which are easily available and which are readily acceptable by the people concern. Such a substance is Potash alum known as "Kyaut-Chin" in Myanmar, which has been used since ancient times for removing turbidity from drinking water. A number of studies on the disinfecting properties of alum have been done(1,2)

but we are not aware of any studies looking into the optimal concentration of alum that has the maximal disinfecting properties and that does not destroy the palatability of water. This is the study of the disinfecting properties of alum in the natural waters and in the experimentally contaminated natural waters.

MATERIALS AND METHODS

Disinfection of natural waters

The study was conducted in Yangon between January to May 1989. The natural water samples were collected from (a) a shallow well in Bahan township used for drinking, (b) a pond in DMR not used for drinking and (c) tap supplied to DMR laboratories from a tube-well which is also used for drinking. The samples were collected in sterile containers by standard methods of collection (3). They were collected in the morning and transported to the laboratory and processed within

one hour at room temperature.

Alum concentration of 0.05% and 0.1% was added to all 3 types of water samples. Total bacterial count (TBC) and faecal coliform count (FCC) were determined on them after 0, 1, 2 and 4 hrs. TBC was done by drop plate method(4). FCC was done by membrane filtration method as described by WHO, 1983(5). These counts were made in the clean supernatant portion of the water samples and not in the bottom precipitates. However, the sediment was inoculated on nutrient agar to test if there were any viable bacteria.

Disinfection of natural waters contaminated with infective dosages of Enterotoxigenic *Escherichia coli* (both LT+ & ST+) *V. cholerae* 01, *S. typhi* and *Shigella dysenteriae* 1.

The waters were seeded with one of the following laboratory strains: ETEC(408-3), *V. cholerae* 01 (Inaba type), *S. typhi* and *Shigella dysenteriae* 1. The test strains were added to each sample of water to obtain initial concentrations of their infective dose: 10^6 /ml - ETEC (6), 10^6 /ml - *V. cholerae* 01 (7), 10^7 /ml - *S. typhi*(8) and 10^7 /ml - *Shigella dysenteriae* 1(9) of untreated water. For maintenance and short term storage all strains were grown in nutrient broth for 24 hrs at 37°C and kept as stock culture at 4°C. After seeding of these strains, each of these varying concentrations of alum (0.025%, 0.05% and 0.1%) were added to each sample of water. All bacterial counts were performed as viable counts by surface inoculation of 0.01 ml of the relevant dilutions on the appropriate agar plates(4) after 0, 1, 2, and 4 hrs.

Samples for bacterial counts were obtained by pipetting off 1 ml of the supernatant dilution, the tip of the pipette being placed 5 cm below the surface. For bacterial count in sediment, this container was shaken vigorously and

the sample was taken. Dilutions were prepared as 10 fold dilutions in normal saline (0.9% NaCl). For bacterial counts the following media were used: Mac Conkey agar for ETEC, Salmonella Shigella agar for *S. typhi* and *Shigella dysenteriae* 1 and Tetrathionate citrate bile sucrose agar for *V. cholerae* 01. Plates were incubated aerobically at 37°C for overnight and viable counts were done.

Statistical Analysis

The effects of different concentrations of alum on bacteria were compared using the log rank test(10). The calculations were made on HP41 CV Calculator using the log rank test for two to four groups(11).

RESULTS

Disinfection of natural waters

When alum concentration of 0.1% was added to the tap water, the water becomes sour and loses its palatability. Therefore, only 0.05% was used in disinfecting the natural waters. At this concentration, the pH of tap water was decreased from 6.8 to 5.1. Within one hour of adding alum, flocculates were formed, and after 2 hrs, sediments could be seen at the bottom of the samples and much more in the pond than other natural waters. The sedimentation process completed within 2-4 hours.

Table 1 shows TBC in the samples with and without alum at different times. These were the average counts tested on 12 samples of each natural water. Alum concentration of 0.05% can decontaminate the total bacterial in natural waters within 2-4 hours. These count differences and decreasing trends were statistically significant at $p < 0.001$. Alum concentration of 0.1% can also decontaminate them within 2-4 hours. Similar results were obtained

Table 1. Survival of total bacteria in natural waters with or without Potash alum

	Mean Total Bacterial Count (CFU ² /ml)			
	0 hr	1 hr	2 hr	4 hr
Pond water	0.9×10^4	0.9×10^4	1.5×10^4	2.1×10^4
Pond water + alum 0.05%*	0.9×10^4	0.9×10^2	0.5×10	0
Shallow well water	1.1×10^4	1.1×10^4	1.8×10^4	2.4×10^4
Well water + alum 0.05%*	1.1×10^4	1.5×10^2	0.9×10	0
Tap water	0.8×10^2	0.8×10^2	1.2×10^2	2.2×10^2
Tap water + alum 0.05%*	0.8×10^2	0.6×10	0	0

*Tests for trend and difference in these groups were statistically significant at $p < 0.001$.

¹Mean of 12 samples result

²CFU = Colony Forming Unit

Table 2. Survival of *V. cholerae* O1 in natural waters containing different concentrations of alum

Time (hrs.)	No alum	Percentage of alum concentration		
		0.025 ^a	0.05 ^a	0.1 ^a
0	2.1×10^4	1.2×10^3	0.2×10^3	0.2×10^2
1	3.3×10^4	1.1×10^2	1.7×10	0
2	2.6×10^4	1.4×10	0	0
4	2.3×10^4	1.1×10	0	0

a = Tests for trend and difference in these groups = $p < 0.001$

for FCC in alum concentrations of 0.05% and 0.1%.

Disinfection of natural waters containing enteric bacterial pathogens

Alum concentrations of 0.05% and 0.1% were found to be most effective in removing *V. cholerae* O1, reducing this bacteria from 10^4 /ml to 0 within 1 hr (Table 2). Likewise, a similar result for Shiga bacillus was obtained within 2 hrs (Table 3).

On the other hand, the effect of alum (0.05%) was not so striking on *S. typhi*. This bacteria decreased 1 log from 10^5 /ml in 1 hr, 2 logs after 2 hrs and 3 logs after 4 hrs (Table 4). But the effect of alum (0.1%) was not so effective on *S. typhi*. Similarly for the ETEC, 2 logs reduction occurred from 10^6 /ml in 1 hr, 3 logs after 2 hrs and 4 logs after 4 hrs, in 0.05% and also in 0.1% concentrations of alum (Table 5).

By resuspension of the sediment V. cholerae 01 and Shiga bacillus were totally decontaminated in the sediment also. But on testing S. typhi and ETEC,

in the reduction of viable bacteria, there was 1 log more decrease in the supernatant water than in the sediment after 2 and 4 hrs.

Table 3. Survival of Shiga bacillus in natural waters containing different concentrations of alum

Time (hrs.)	No alum	Percentage of alum concentration		
		0.025 ^a	0.05 ^a	0.1 ^a
0	2.1×10^4	1.2×10^3	0.2×10^3	0.2×10^2
1	2.3×10^4	1.1×10^2	1.7×10	0
2	2.4×10^4	1.4×10	0	0
4	2.3×10^4	1.1×10	0	0

a = Tests for trend and difference in these groups = $p < 0.001$

Table 4. Survival of S. typhi in natural waters containing different concentrations of alum

Time (hrs.)	No alum	Percentage of alum concentration		
		0.025 ^a	0.05 ^a	0.1 ^a
0	3.8×10^5	5.2×10^4	2.8×10^4	1.4×10^4
1	4.2×10^5	2.1×10^3	1.9×10^3	1.5×10^3
2	4.1×10^5	0.3×10^3	0.4×10^3	0.1×10^2
4	4.3×10^5	0.1×10^3	0.4×10^2	0.1×10^2

a = Tests for trend and difference in these groups = $p < 0.001$

Table 5. Survival of ETEC (both SI+ & LI+) in natural waters containing different concentrations of alum

Time (hrs.)	No alum	Percentage of alum concentration		
		0.025 ^a	0.05 ^a	0.1 ^a
0	3.8×10^8	3.2×10^8	3.2×10^8	2.4×10^8
1	4.2×10^8	2.1×10^6	1.9×10^6	1.5×10^6
2	4.1×10^8	0.3×10^6	1.4×10^5	0.8×10^5
4	4.3×10^8	0.1×10^5	0.2×10^4	0.5×10^3

a = Tests for trend and difference in these groups = $p < 0.001$

DISCUSSION

Although there are a number of studies on the effect of alum on different bacteriae(1,2), we are not aware of any studies looking into its effect on the principal enteric pathogens using different concentrations in different types of water.

We found that the decontamination power of alum was best at 0.05% and 0.1% concentrations in natural waters. However, at 0.1% it causes sour taste to the natural waters and therefore changes their palatability. Even so, the effect of alum (0.1%) was not so effective on *S. typhi* in natural waters. It decreased 1 log only from 10^5 /ml in 1 hour and also after 2 and 4 hours. For this reason the use of 0.05% solution is recommended for use in future intervention studies to improve household water.

In spite of the fact that alum consistently reduces the number of different bacteriae in water, its effectiveness seems to depend on the nature and the load of bacteria. It is most effective against *V. cholerae* 01 and *S. dysenteriae* 1 which are susceptible to low pH. Alum seems to kill *V. cholerae* 01 and *Shiga bacillus*, rather than expedite their sedimentation for, as opposed to other tested bacteriae (*S. typhi* and ETEC), they cannot be isolated from the sediment. Depending on the initial bacterial load present in natural waters, it takes more time to totally clear TBC and FCC. This effect is strikingly seen with *S. typhi* and ETEC in which the initial seeding doses (infective doses) are much higher than other bacteriae. Moreover, this limited effect may probably be due to their ability to survive in acidic medium (12, 13).

Despite its effect on *S. typhi* and ETEC, alum can probably be used in household decontaminating interventions. This is because 1) like in Bangladesh 2) potash alum has been in use in

Myanmar since ancient times to clear turbid water and most of the people in Myanmar know and accept this method of treatment; 2) it is easy to apply; 3) is cheap (only 1 kyat worth is required daily to decontaminate on average household of 5 persons using water at 20 litres/cap/day); and 4) is readily available.

Even if it is not used as an intervention measure, it can be used with an advantage in treating drinking water during cholera outbreaks (2) and probably also in bacillary dysentery epidemics. It may be used for chlorine in high risk groups like victims of natural disasters, fires and for refugees, if chlorine is not available.

ACKNOWLEDGEMENT

We would like to express our appreciation to Dr. U Khin Maung Tin for his advice, to carry out this study.

REFERENCES

1. Ahmed, K., Jahan, K. & Hug, I. Decontamination of drinking water by alum for the preparation of oral rehydration solution. *Food and Nutrition Bulletin*. 1984, June, 6(2): 54-57.
2. Khan, M.U. Preventing the transmission of cholera using alum potash. *Annual Report*, ICDDR B 1985; 16.
3. Cruickshank, R. Examination of water, milk and food. 11th ed. (revised reprint) *Medical Microbiology*. 1970; 962.
4. Miles, A.A. & Misra, S.S. the estimation of the bactericidal power of the blood. *Journal of Hygiene*. 1938; 38: 732-749.
5. World Health Organization. *Guidelines for drinking water quality Vol. 3. Drinking water quality control in small community*

- supplies. Geneva: WHO. 1984; 3: 112-113.
6. Dupont, H.L., Formal, S.B., Hornick, R.B. et al. Pathogenesis of *E. coli* diarrhoea. New England Journal of Medicine. 1971; 285: 1.
 7. Spira, W.M., Khan, M.U., Saeed, Y.A. & Sattar, M.A. Microbiological surveillance of inter-neighbourhood EL Tor cholera transmission in rural Bangladesh. Bulletin WHO. 1980; 58(5): 731-740.
 8. Blaser, M.J. & Newman, L.S. A review of human salmonellosis: 1. Infective dose. Review of Infectious Diseases. 1982; 4: 1096-1106.
 9. Dupont, H.L. & Pickering, L.K. The response of man to virulence *Shigella flexneri* 2A. Journal of Infectious Diseases. 1969; 119: 296-299.
 10. Peto, R., Pike, M.C., Armitage, P., et al. Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II Analysis and examples. British Journal Cancer. 1977; 35: 1-39.
 11. Abramson, J.H. & Peritz, E. Calculator programs for the health sciences. Oxford: Oxford University Press. 188 & 306.
 12. Cruickshank, R. Physical and Chemical Methods I. 11th ed. (revised reprint) Medical Microbiology. 1970; 846.
 13. Goverd, K.A., Beech, F.W., Bobbs, R.P. & Shammen, R. The occurrence and survival of coliforms and Salmonellas in Apple Juice and Cider. Journal of Applied Bacteriology. 1979; 46: 521-530.

Accepted for publication 13 June 1990.

Some variations in membrane proteins of erythrocytes obtained from subjects with thalassaemia trait and G-6-PD deficient genes

*Myint-Oo, **Myat-Phone-Kyaw & ***Thein-Than

*Parasitology Research Division, ***Biochemistry Research Division, DMR
**Institute of Medicine (2), Yangon

A total of 350 erythrocyte samples were screened for haemoglobin variant, G-6-PD deficiency and thalassaemia trait genes. Of these, 104 samples were subjected to membrane protein analysis using SDS-PAGE electrophoresis and double staining with silver and Coomassie Blue. Membrane protein patterns of erythrocytes from subjects with a single genetic defect (i.e. either G-6-PD deficient or thalassaemia trait) were found to be similar to that of normals but those from subjects with double genetic defects (i.e. combination of G-6-PD deficiency and thalassaemia trait) had a significantly reduced content of spectrin, band 3 and glycophorin A.

INTRODUCTION

Erythrocyte membrane proteins are divided into two major classes, peripheral and integral. Integral proteins penetrate or span the lipid bilayer and interact with the hydrophobic lipid core. The most abundant red cell transmembrane proteins are Band 3 and carbohydrate rich glycoproteins.

Peripheral proteins are not inserted into the bilayer but reside on one surface. They are bound to the membrane by interaction with integral proteins or the polar head of the lipid bilayer. The major peripheral proteins, located on the cytoplasmic side of the membrane form the cytoskeletal network which consists mainly of spectrin, band 4.1, actin and band 6. [Figure 1] (1).

It has been reported that subjects with various types of Hereditary spherocytosis(HS)(2) and Hereditary Pyropoikilocytosis(HPP)(3) demonstrated deminished amount of spectrin. There is also evidence that Band 3 was decreased in erythrocytes with sickle cell disease (4).

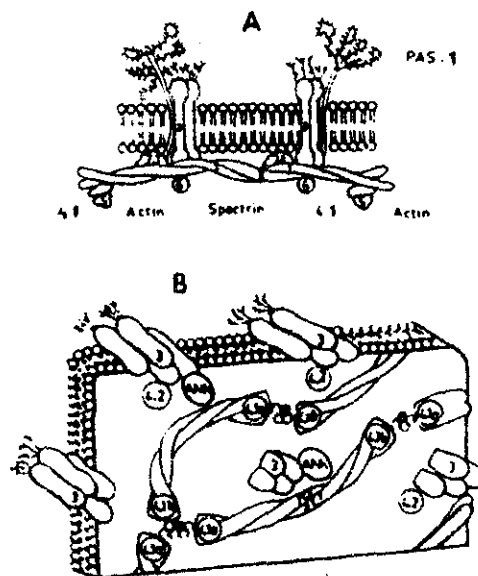


Fig.1. Representation of the erythrocyte membrane with particular emphasis on the proteins that comprise the cytoskeleton. A. Highly schematic illustration of the organization of the red cell membrane. B. Model of the red cell membrane as viewed from the interior of the cell. The numbers correspond to the numbering given in fig.1. Ank=ankyrin. Based on Goodman & Shiffer(1983).

The hereditary red cell disorders like the abnormal haemoglobins, thalassaemia trait genes and G-6-PD deficiency are common in Myanmar. The prevalence of Haemoglobin E is 21-28% in the Burmese(5), β -thalassaemia trait is 4.3% (6) and α -thalassaemia trait is 2-6%(7). Also, the incidence of G-6-PD deficiency (hemizygote) was reported to be 9% in Burmese males(8).

This study was carried out to see whether additional polymorphisms of membrane proteins and glycoproteins in erythrocytes with G-6-PD deficiency and thalassaemia trait genes, either singly or in various combinations could be observed in an attempt to gain some insight into the variation of the cytoskeletal network in those erythrocytes.

MATERIALS AND METHODS

Subjects:

Venous blood samples in Acid-Citrate-Dextrose (ACD) (1:4) were collected from 350 random subjects (male only) coming to Blood bank, Yangon General Hospital or the Defense Services General Hospital, Mingaladon.

Screening of abnormal haemoglobins and thalassaemia trait:

Red cell indices, osmotic fragility, Haemoglobin A₂ measurements, Haemoglobin F estimation, serum iron determination, starch gel electrophoresis and cellulose acetate electrophoresis were carried out according to the standard methods by Dacie and Lewis(9).

Thalassaemia trait and other abnormal haemoglobins were classified using the flow chart described by Modell and Berdoukas(10).

Screening and Genotyping of G-6-PD variants:

Screening of G-6-PD variants and genotypes were done according to the standard methods by WHO (11) and cytochemical assays(12).

Analysis of red cell membrane:

The preparation of red cell membrane proteins for SDS-PAGE and double staining with silver Coomassie Blue were carried out according to the method of Dzandu et al.(13) and analysed according to a schematic representation based on that of Gratzer (14) [Figure 2]. Quantitation of protein bands was achieved using a densitometer and estimating the areas under the respective curves.

RESULTS

A summary of the samples used for red cell membrane analysis using SDS-PAGE is given in Table 1. Of 285 normal control samples, only 39 were used for red cell membranes fractionation. Membrane analysis was also carried out on 24 subjects with G-6-PD deficiency (mild type; Gd^{B-}) gene, 10 with severe G-6-PD deficiency (Gd⁻ Myanmar), 20 with β -thalassaemia trait, 8 with β -thalassaemia trait and G-6-PD mild deficiency, and 3 with β -thalassaemia trait plus G-6-PD severe deficiency.

Electrophoretic patterns of membrane proteins of erythrocytes from 104 blood donors with various types of G-6-PD deficiency and β -thalassaemia traits were analysed by densitometry (Figure 3). No significant differences between the membrane protein components of normal red cells and those of red cells from subjects with single genetic defects of either G-6-PD deficiency (mild or severe) or β -thalassaemia traits were observed. However, there

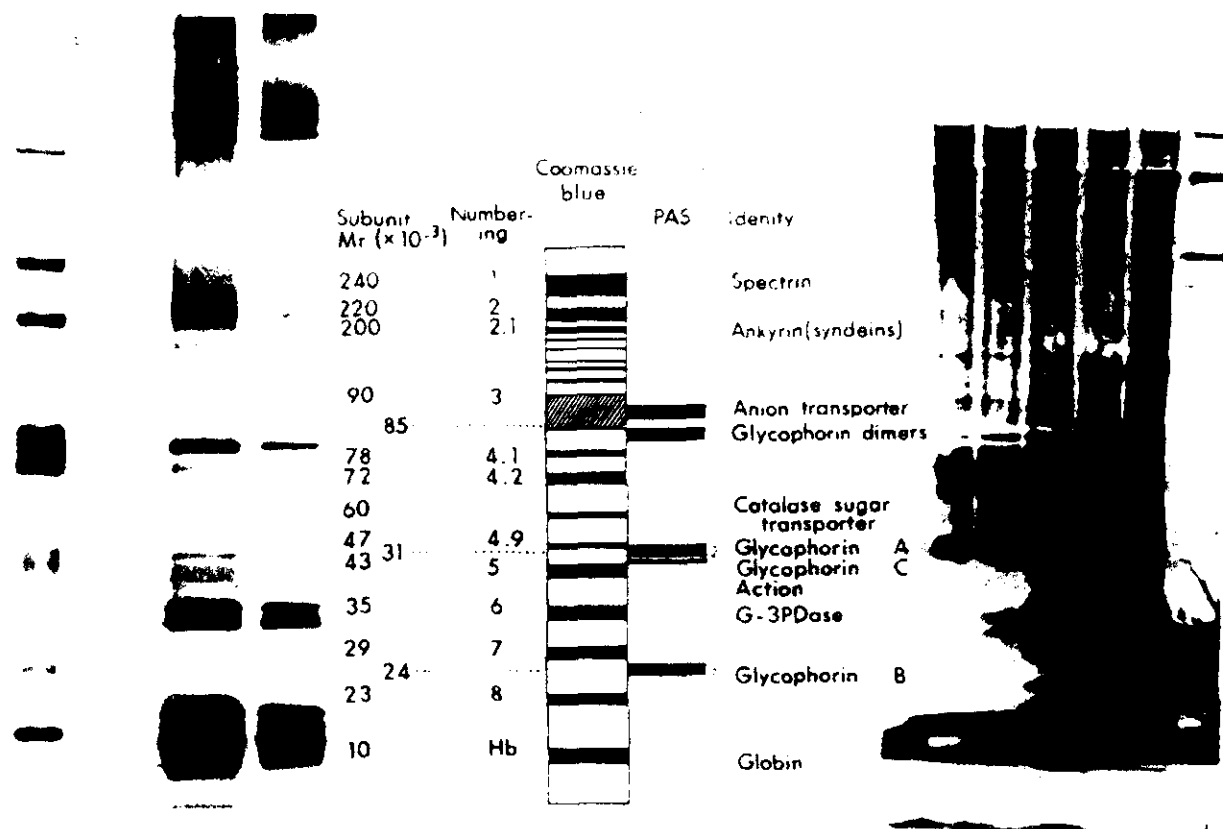


Figure 7. Schematic representation of erythrocyte membrane proteins of the human red cell separated by SDS-PAGE.

were significant reductions in spectrin and band 3 proteins of red cells collected from subjects with double genetic defects of β -thalassaemia trait with either G-6-PD mild deficient gene or severely deficient genes ($P < 0.01$).

The sialoglycoproteins (glycophorins) variations were also observed in membranes of red cells obtained from subjects with β -thalassaemia trait and G-6-PD deficiency (Table 2). Significant differences were recorded in glycophorin A (α_1 and α_2) values scanned by densitometer ($P < 0.01$).

DISCUSSION

It has been reported that SDS-PAGE analysis in membrane proteins of erythrocytes from patients with various

types of Hereditary Spherocytosis(HS) and Hereditary Pyropoikilocytosis(HPP) were all partially deficient in spectrin with a range of 74,000-200,000 copies (2).

The spectrin molecule is a dimer composed of two non-identical subunits now generally referred to as α and β rather than band 1 and 2. In our study, a significant reduction of spectrin, especially β -sub unit, was recorded in erythrocyte membrane proteins of subjects with β -thalassaemia trait and G-6-PD deficiency.

Band 3 membrane protein is decreased and split into two or three overlapping components in red cells with haemoglobin S(4). In ovalocytosis, the major cytoskeletal protein, spectrin appeared normal as judged by its extractability.

Table 1. Number of cases completed for red cell membrane analysis

Red cell genetic defects	Samples completed for study	
	Number	%
<u>G-6-PD deficient genes</u>		
Mild type (Gd ^{B-})	24	6.86
Severe type (Gd ^{-Myanmar})	10	2.86
<u>Thalassaemia trait genes</u>		
β -thalassaemia trait gene	20	5.72
<u>Double genetic defects</u>		
Gd ^{B-} with β -thal. trait	8	2.29
Gd ^{-Myanmar} with β -thal. trait	3	0.86
<u>Normal gene</u>		
G-6-PD Normal (Gd ^{B+}) with no haemoglobinopathy	285*	81.43
	350	

* Only (39) samples were analysed

and the thermal stability of solubilized spectrin that reflects as normal erythrocytes(15).

In β -thalassaemia trait, the impaired ratio of β to α chain synthesis and precipitation of unpaired α -chains, leads to inclusion bodies which interact with the membrane leading to localized rigidity and thus a decrease in deformability. When these heinz bodies are present in cells they may be selectively removed during their passage through the spleen by pitting or plucking of the inclusion. This results in loss of the surrounding membrane materials, decreased surface area to volume ratio and contributes further to reduce deformability and cell survival(16). In the present study, the reduction of glycophorin a dimers could reflect the reduction of band 3 and spectrin of red cell membranes obtained from subjects with double genetic defects (i.e. β -thalassaemia trait gene and G-6-PD deficient Gd^{-Myanmar} gene).

On the other hand, in G-6-PD deficient erythrocytes the hexose monophosphate (HMP) shunt activity is significantly decreased and it might be expected that the shape recovery rate of G-6-PD deficient cells would be decreased(17). In HMP, G-6-PD enzyme occupies a key position in which the reduction of nicotinamide adenine dinucleotide phosphate(NADP) to NADPH; yet NADP is required by the erythrocyte to maintain glutathione(GSH) in the reduced state and furthermore, it may play an important role in the catabolism of hydrogen peroxide (H_2O_2). G-6-PD deficient cells are low in glutathione reductase (GSSG) (18) and accumulation of H_2O_2 occurred leading to morphological changes characterized by increased echinocytic formation(19). In addition, decreased cell deformability commensurate with increased membrane rigidity was found. Both changes are closely related with the extent of spectrin haemoglobin cross-linkage and the alteration in membrane function by

Table 2. Variation in glycoporphins of red cell membranes obtained from subjects with different genetic defects

Area under curve (mm ²) as densitometer graph						
Sialoglycoprotein Bands	Normal	Single genetic defects			Double genetic defects	
	Gd ^{B+} with no thal. trait (mean±SD) (n = 39)	Gd ^{B-} with no thal. trait (mean±SD) (n = 24)	Gd-Myanmar with no thal. trait (mean ± SD) (n = 10)	Gd ^{B+} with B - thal. trait (mean ± SD) (n = 20)	Gd ^{B-} with B -thal. trait (mean ± SD) (n = 8)	Gd-Myanmar with B-thal. trait (mean ± SD) (n = 3)
Glycophorin A dimer (α ₂)	50.0 ± 2.1	46.7 ± 1.8	43.8 ± 1.9	47.9 ± 1.5	28.2 ± 1.4 ^S	27.5 ± 1.3 ^S
Glycophorin A & B (α δ)	13.0 ± 1.4	12.4 ± 1.5	10.9 ± 0.9	14.1 ± 1.1	10.0 ± 1.1	10.2 ± 0.6
Glycophorin A monomer (α)	8.0 ± 1.9	7.6 ± 1.7	5.3 ± 0.8 ^S	6.9 ± 1.1	5.0 ± 0.6 ^S	5.2 ± 0.6 ^S
Glycophorin C (γ)	12.1 ± 1.7	11.7 ± 1.6	11.9 ± 1.4	11.6 ± 0.7	12.8 ± 0.7	12.3 ± 1.2
Glycophorin B monomer (δ)	18.6 ± 2.1	17.9 ± 1.2	18.1 ± 0.3	18.1 ± 0.8	19.0 ± 0.8	16.5 ± 0.5

S = Significant at $p < 0.01$. Significant of difference was calculated against normal values using students "t" test.

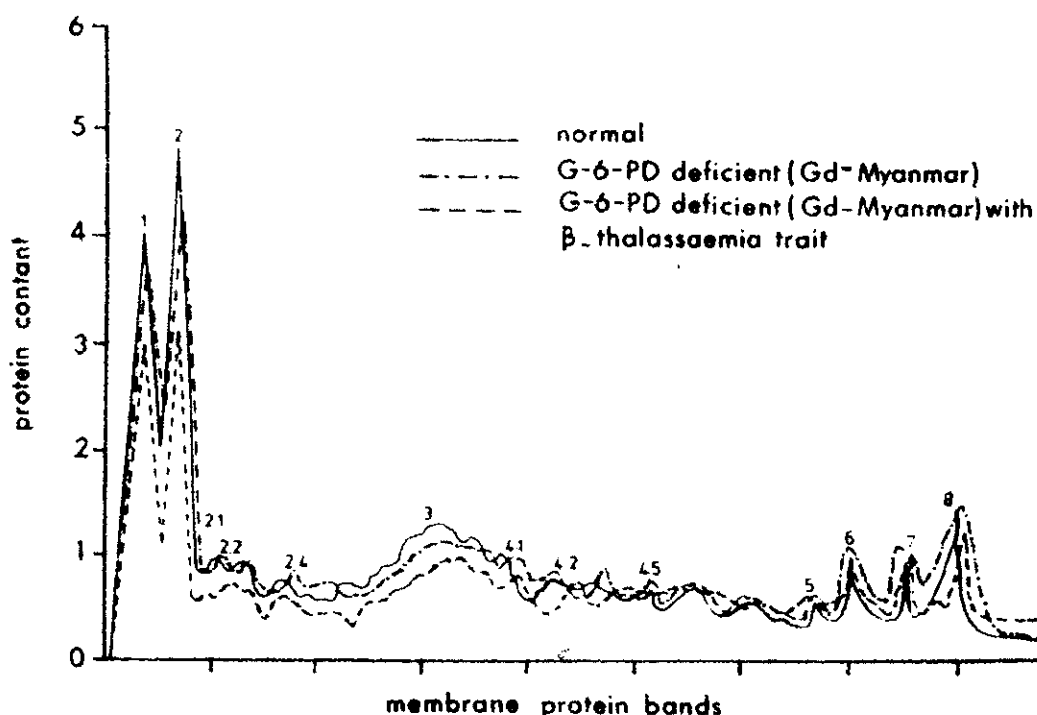


Figure 3. Membrane proteins of normal, G-6-PD deficient (Gd-Myanmar) and G-6-PD deficient (Gd-Myanmar) with β -thalassaemia trait. Gels were loaded with equal amount of membrane protein

spectrin haemoglobin aggregations.

Therefore, the combination of two genetic defects in a group of red cells altered the components of membrane proteins in these red cells. The effect of excess α -chain precipitates might combine with spectrin-haemoglobin cross-linking by the accumulation of H_2O_2 leading to a reduction in spectrin and band 3 in membrane proteins of red cells with both thalassaemia trait gene and G-6-PD deficient gene.

ACKNOWLEDGEMENTS

This study was supported by the long-term Institutional Strengthening Grant from WHO/TDR, Geneva. The authors would like to express their gratitude to Dr. Aung-Thun-Batu, Director, WHO/SEARO, New Delhi, India and Dr. Khin-Maung-Tin, retired Director-

General, Department of Medical Research, Ministry of Health, Yangon, Myanmar for their encouragements to carry out this study. The authors also like to thank Lt. Col. Tin-Maung-Aye, Pathologist, D.S.G.H. (1), Mingaladon and all the staffs of Blood Banks of D.S.G.H. and Y.G.H. This work was done by utilizing facilities donated by JICA.

REFERENCES

1. Goodman, S.R., Shiffer, K.A., Casoria L.H. & Eyster M.E. Identification of the molecular defect in the erythrocyte membrane skeleton of some kindreds with hereditary spherocytosis. *Blood*. 1982; 60(3): 772-784.
2. Arge, P., Casella J.F., Zinkham W.H., McMillan C. & Bennett, V.

- Partial deficiency of erythrocyte spectrin in hereditary spherocytosis. Nature. 1983; 314: 380-383.
3. Liu, S.C., Palek, J. Prchai, J.T. & Castleberry, R.P. Altered spectrin dimer-dimer association and instability of erythrocyte membrane skeletons in hereditary pyropoikilocytosis. Journal of Clinical Investigation. 1981; 68: 597-605.
 4. Riggs, M.G. & Ingram, V.M. Differences in erythrocyte membrane proteins and glycoproteins in sickle cell disease. Biochemical and Biophysical Research Communication. 1977; 74(1): 191-197.
 5. Aung-Than-Batu & Hla-Pe. The incidence of HbE. Tropical and Geographical Medicine. 1970; 23: 15-18.
 6. Aung-Than-Batu, Khin-Kyi-Nyunt & Hla-Pe. The thalassaemia in Burma. Union of Burma Journal of Life Science. 1968; 1: 241-247.
 7. Aung-Than-Batu, Hla-Pe & Khin-Kyi-Nyunt. The incidence of alpha-thalassaemia trait. Tropical and Geographical Medicine. 1971; 23: 23-24.
 8. Aung-Than-Batu & Hla-Pe. Glucose-6-phosphate dehydrogenase deficiency in Burma. Union of Burma Journal of Life science. 1969; 2: 59-61.
 9. Dacie, J.V. & Lewis, S.M. Practical Haematology. 1984; 52-188. 6th ed. Edinburgh, London, Melbourne and New York: Churchill Livingstone.
 10. Modell, B. & Berdoukas, V. The clinical approach to thalassaemia. 1984; Chapter 1: 1-25. London, Tokyo, Harcourt Brace Favanovich Published, Grune & Stratton.
 11. World Health Organization. Technical Report Series No. 366. Standardization of Procedures for the Study of Glucose-6-Phosphate. 1967.
 12. Van-Noorden, C.J.F. & Vogels, I.M.C. A sensitive cytochemical staining method for glucose-6-phosphate dehydrogenase activity in individual erythrocytes. British Journal of Haematology. 1985; 60: 57-63.
 13. Dzandu, J.K. Deh, M.E., Baratt, D.L. & Wise, G.E. Detection of erythrocyte membrane protein sialoglycoproteins and lipids in the same polyacrylamide gel using a double staining technique. Proceedings of the National academy of Sciences in U.S.A. 1984; 81: 1733-1737.
 14. Gratzner, W.B. The red cell membrane and cytoskeleton: The cytoskeleton of red blood cell. Biochemical Journal. 1981; 198: 1-8.
 15. Saul, A. Ovalocytosis and resistance to malaria. The Queensland Institute of Medical Research, 39th annual report of molecular Biology and Parasitology. 1984; 48: 1983-1984.
 16. Rice-Evans, C.A. & Dunn, M.J. Erythrocyte deformability and disease. TIBS. 1982; 282-285.
 17. Alhanty, E., Synder M. & Sheetz, M.P. Glucose-6-phosphate dehydrogenase deficient erythrocytes have an impaired shape recovery mechanism. Blood. 1984; 65(5): 1198-1202.
 18. Friedman, M.J. Inherited Resistance to Malaria. Biochemical and Physiology of Protozoa. 1981; 2nd ed., 4: ed. by Leoandowsky M. and Hunter S.H., New York.
 19. Synder, L.M., Fortier, N.L. Trainor, J., Jacobs, J., Leb, L., Lubin, B., Chiu, D., Shohet, S. & Mohandas, N. Effect of hydrogen

peroxide exposure on normal human erythrocyte deformability, morphology, surface characteristics and spectrin-haemoglobin cross-linking.

Journal of Clinical Investigation.
1985; 76: 1971-1977.

Accepted for publication 13 June 1990.

"DMR-larval trap" for possible control of Aedes aegypti(L), the principal vector of DHF in Yangon

Anthony Sebastian & Myat-Myat-Thu

Medical Entomology Research Division,
Department of Medical Research

The Department of Medical Research (DMR) has developed a larval/pupal trap with a view to control Aedes (Stegomyia) aegypti (L) effectively and economically. "DMR-larval trap" is a mechanical device and is capable of trapping all stages of larvae and pupae and that the number of aquatic stages trapped is directly proportional to the number of larvae and pupae present in the container-habitat. The trap is also capable of trapping greater number of late stages than the earlier ones which is more favourable from control point of view. It is found to trap about 47.85% of all larvae and pupae within the first 24 hours achieving complete control within 10 days with sustained control thereafter.

INTRODUCTION

Dengue haemorrhagic fever (DHF) has been prevalent in Yangon since 1970 with recurrence every year. The mosquito vector is Aedes (Stegomyia) aegypti (L) which breeds primarily in metal drums (67.5%), Bago jars (20.06), and earthen pots (4.98%) all containing stored water meant for daily domestic use. In Yangon, the public are accustomed to storing domestic water mostly in metal drums, Bago jars, earthen pots and other containers and Ae. aegypti(L) breeding is well established in them.

Seasonal surveys of all breeding habitats of Yangon Ae. aegypti(L), indoors and outdoors, indicate over 90% breeding in domestic water storage containers and the remaining 2% in miscellaneous receptacles (2%) and in antguards (0.2%).

Of all the domestic water storage containers, metal drums, Bago jars and earthen pots were found to support

92.5 per cent of all the aquatic stages produced by all A. aegypti(L) sources in Yangon(1). It follows that if these three major producing types of container-habitats can be checked to prevent the prolific breeding by mechanically flushing the containers of the remnant water and debris with larvae and pupae before refilling with a fresh supply at weekly intervals, over 90 per cent control can be ensured.

In view of controlling A. aegypti(L) economically and effectively, the Medical Entomological Research Division of the Department of Medical Research, Yangon, as part of its programme of work, has developed a larval/pupal trap ('DMR-larval trap') and this paper first of its series, describes the trap and its performance in trapping A. aegypti(L) larvae and pupae.

MATERIALS AND METHODS

Description of 'DMR-larval trap'

It is a mechanical device and consists

of a 15 cm diameter plastic receptacle, 6.25 cm deep with a 10 cm invaginated base to form a stumpy inverted funnel (5 cm) high, tapering to (1.25 cm) diameter spout at the centre (Fig. 1). The level of the spout is purposely made to a height of (5 cm) so that it falls short by (1.25 cm) from the brim of the receptacle. Thus, when the trap is set, the spout remains always immersed (1.25 cm) from the surface of water. The entire device is made of plastic and as the specific gravity of plastic is less than one, the receptacle will therefore remain buoyant with only the brim of the receptacle protruding out of the surface of water when immersed to trap.

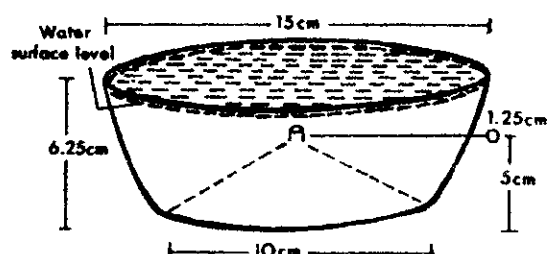


Figure 1. Diagrammatic representation of 'DMR-larval trap'

Trapping mechanism

Larvae and pupae of Aedes aegypti(L) at all stages of growth come up to the water surface, with their respiratory siphon in contact with the surface film for the intake of oxygen and after a short period of rest glide down in search for food particles.

Movements of the larvae are very characteristic. Christopher(2) draws attention to the extreme restlessness of the larvae of Aedes aegypti(L) continually roaming through the container-habitat. Of various reactions of the larvae to stimuli, that of negative phototropism is especially characteristic of the larvae of Aedes aegypti(L). Shanon and Putnam(3) while describing

the behaviour of the larvae, noted as especially characteristic, of its extreme restlessness, strong negative phototropism and extreme sensitiveness to vibrations and to light. Invariably, in all circumstances and in all stages of growth, the larvae at once begin to swim away from the light and accumulate in that part of container which is remote from the source of light.

Taking all these behaviouristic features of Aedes aegypti(L) into consideration, the 'DMR-larval trap' was designed and may successfully be set by placing the trap at the site of accumulation of aquatic stages for maximum trapping.

The larvae and pupae present near the trap wriggle up and brouse along the sides any the invaginated base and ultimately find their way into the receptacle through the spout. After entering, the larvae invariably swim to the side of the receptacle and thus get trapped. However, some of them wriggle up to the spout and dive down immediately or after taking in oxygen at the water surface and escape. A slight modification in the shape and size of the spout can perhaps prevent the trapped larvae from escaping. Also changes in the shape and size of the trap (receptacle) to get maximum trapping at an optimum size may need to be determined. It is intended that these determinations and modifications will be made and reported in the second series of report on 'DMR-larval trap' in due course of time.

Container-habitat used in the study

Since maximum breeding of Aedes aegypti(L) in Yangon occurs in metal drums, this container-habitat was chosen and used in the study on the trapping performance of 'DMR-larval trap'. Metal drum is a cylindrical barrel 36.8 inches (93.47 cm) high with a radius of 11 inches (27.94 cm) and a capacity of 44 US gallons.

Aedes aegypti(L) aquatic stages used

Aedes aegypti(L) breeding in metal drums under field conditions were collected and a required number were counted and used.

A known number of all aquatic stages of *Aedes aegypti*(L) were counted and released in a drum full of tap water. An hour after the release, the larvae and pupae get adapted to the new environment when the 'DMR-larval trap' was set at the site where the larvae accumulated most. The trap was left for a specified duration at the end of which, the trapped larvae and pupae were removed and counted.

RESULTS AND DISCUSSION

Within a few minutes after the setting of the 'DMR-larval trap' larvae and pupae were seen entering the trap with a negligible number escaping initially. But as the number of the trapped larvae increased, they got scattered all over the receptacle including the vicinity of the spout, when the number of escaping larvae gradually increased. The number of entering larvae in turn depended on the density of aquatic stages present in the container-habitat. The higher the density, the greater were the number of entering larvae.

Table (1) shows the number of *Aedes*

Table 1. Trapping capacity of 'DMR-larval trap' in relation to different larval and pupal counts during different trapping periods

No. of aquatic stages exposed for trapping	Duration of trapping period	Number of replicates	Average no. of aquatic stages trapped
2,000 all stages	1 hr (during day)	3	509 $\pm$ 79.05
- do -	2 hr (")	"	535 $\pm$ 57.13
- do -	3 hr (")	"	679 $\pm$ 69.21
- do -	17 hr (overnight)	"	491 $\pm$ 44.03
1,000 all stages	1 hr (during day)	3	243 $\pm$ 37.58
- do -	2 hr (")	"	320 $\pm$ 29.19
- do -	3 hr (")	"	353 $\pm$ 33.01
- do -	17 hr (overnight)	"	364 $\pm$ 33.08
500 all stages	1 hr (during day)	3	157 $\pm$ 6.81
- do -	2 hr (")	"	190 $\pm$ 10.02
- do -	3 hr (")	"	198 $\pm$ 14.30
- do -	17 hr (overnight)	"	140 $\pm$ 22.74

aegypti(L) aquatic stages trapped in relation to different larval and pupal counts at different trapping periods. It is obvious from the table that the number of larvae and pupae trapped is directly proportional to the number of larvae and pupae present in the container-habitat.

The average number of trapped aquatic stages during an hour, as can be seen from the table is fairly high and increases with the increase in the trapping period though not proportionately. It is also apparent from the table that hourly trapping can effect control of aquatic stages within a shorter period than trapping every 2 or 3 hourly. But hourly trapping for purposes of control eventually by the public may not be possible. A minimum of 3 hour or more trapping period once a day is likely to be more conducive for a house-wife to remove the trap and dispose the contents of the trapped larvae and pupae on earth and re-set the trap for another 3 hour or so.

The overnight trapping figures in Table 1, are rather low despite much longer trapping period of 17 hours. This is

because the larvae and pupae were scattered all over the habitat due to the absence of light and as a result the chances of their coming in contact with the trap were less as opposed to those trapping during the day under the influence of phototrophism.

In order to find the differences in the number trapped for different aquatic stages, equal numbers for each of the four larval stages and pupae were counted and released in the container. The number trapped during different trapping periods were taken and the results are summarised in Table 2.

It will be observed from the table that greater number of late stages get trapped than the earlier ones and this is simply because the late instars are more active and fast moving than the earlier younger ones. The trapping of late instars mostly is more favourable from control point of view as their elimination can prevent adult emergence. Nevertheless, the trap is capable of trapping all stages ultimately.

A continuous trapping was conducted on 2,000 aquatic stages of Aedes aegypti

Table 2. Trapping capacity of 'DMR-larval trap' on larval stages I-IV and pupae in relation to different trapping periods

Number and stages of larvae and pupae exposed for trapping						Trapping period	No. of Repli- cates	Average number trapped for each stage					All stages mean	S.D
I	II	III	IV	Pupae	Total			I	II	III	IV	Pupae		
200	200	200	200	200	1,000	$\frac{1}{2}$ hr	3	3	8	29	60	94	194 $\pm$ 17.7	
"	"	"	"	"	"	1 hr	"	5	15	63	85	131	299 $\pm$ 27.1	
"	"	"	"	"	"	2 hr	"	3	9	59	120	110	301 $\pm$ 41.9	
"	"	"	"	"	"	3 hr	"	8	31	81	127	79	326 $\pm$ 105.9	
"	"	"	"	"	"	17 hr (over- night)	"	6	20	35	76	54	191 $\pm$ 15.7	

(L) to find the percentage of control achieved within every successive 24 hours. Results are tabulated in Table 3.

From Table (3), it will be observed that during the first 24 hours the control achieved was 47.85 per cent and on the successive days the trapped percentages were much lower due to less number of aquatic stages left in the container. The overall control achieved during the 10 day period of trapping was 89.90 per cent and the number left over were found to be 10. The missing 192 probably died due to larval pathogens present in them.

From the foregoing findings it is apparent that the use of the 'DMR-larval trap' in major breeding sources like metal drums, Bago jars and other container-habitats of Aedes aegypti(L) is likely to achieve high rate of mechanical control effectively and economically.

ACKNOWLEDGEMENT

The authors wish to thank the staff of the Medical Entomology Research Division for all the help given during the course of the study.

REFERENCES

1. Myat-Myat-Thu. Studies on the biology and ecology of Aedes aegypti(L) in Rangoon area. M.Sc. (Zoology) Thesis, Rangoon University. 1975; 54-62.
2. Christophers, S.R. Aedes aegypti (L). The yellow fever mosquito,

Table 3. Percentage of control achieved by the use of the 'DMR-larval trap' in a container infested with 2,000 Aedes aegypti(L) aquatic stages

No. of <u>A. aegypti</u> (L) aquatic stages present in the container	Number trapped 24 hourly	Trapped percentage 24 hourly	Day
2,000 all stages	957	47.85	1
	315	15.75	2
	220	11.00	3
	113	5.65	4
	76	3.80	5
	64	3.20	6
	15	0.75	7
	15	0.75	8
	17	0.85	9
	6	0.30	10
Total	1798	89.90	10

its life history, bionomics and structure. Cambridge University Press, London. 1960; 144-145.

3. Shanon, R.C. and Putnam, P. The biology of Stegomyia under laboratory condition. I. The analysis of factors which influence larval development. Proc. Ent. Soc. Wash. 1934; 36: 185-216.

Accepted for publication 13 June 1990.

Lepromin reaction in patients with falciparum malaria

Tin-Shwe & Pe-Than-Myint

Clinical Research Unit. (Cerebral and complicated Malaria)
Department of Medical Research
Department of Medicine, Institute of Medicine (1), Yangon

The study was done to find out if there is any relation between leprosy and malaria, and to find out the cellular responses of the host to *Mycobacterium leprae* extract in malaria patients. As a preliminary step lepromin was tested on malaria patients. 57.7% of malaria patients were lepromin negative or doubtful reaction. Among the controls only 7% showed negative or doubtful reaction.

INTRODUCTION

The pathological and clinical manifestations of leprosy form a spectrum from lepromatous leprosy (LL) to tuberculoid leprosy (TT). Ridley and Jopling classified them as TT, BT, BB, BL, LL. The factor which mainly determines the place of an individual patient disease in this spectrum is the extent to which cell mediated immunity is expressed. In TT cell mediated immunity and hypersensitivity are well developed (Lepromin reaction strongly positive) whereas in LL they are absent (lepromin reaction negative). Thus leprosy patients may be regarded as a good model to study the immunological response to infection. Hence it is important to study the concurrent infections of malaria and leprosy.

In actual practice it is not ethical to omit Dapsone (DDS) [which has some antimalarial action] among leprosy patients. Hence lepromin reaction was tested on malaria patients.

PATIENTS AND METHODS

116 *P. falciparum* malaria infected patients who were admitted to Thayarwady Civil Hospital during 1986 malaria season were studied. 0.1 ml lepromin supplied from NHDC Carville La 70721 (lepromin A 30/40 million

Bac/ml Lot No. V 97) was injected intradermally on the day of admission to hospital and the site was examined in 3-4 weeks (Mitsuda reaction) for a palpable nodule whose diameter was measured and graded (negative to 3 + reaction) as described in leprosy for students of medicine Bryceson & Pfaltzgraff(1). 100 contacts of malaria patients (family members of malaria patients who were attending the patients at the hospital) were also included in the study as controls. The parasite density of the patients were recorded according to lograthmic scale of Bruce Chwatt(2). The clinical status of the patients were also recorded as Category: 1. Patients with no complications

2. Patients with complications such as high level of parasites in blood (>5% of RBC parasitised, or hyperpyrexia, or with jaundice, or with severe anaemia (PCV <30%)
3. Patients with cerebral signs and symptoms or in deep unrousable coma.

RESULTS

Among malaria patients 86 were males and 30 females of ages between 12-65 years. Of these 67 patients (57.7%)

showed negative or doubtful reaction. 30 patients (25.8%) showed strongly positive (2+ and 3+) reaction (palpable nodule over 5 mm + ulceration).

Among 100 controls 7 subjects (7%) showed negative reaction and 89 or 89% showed strongly positive reaction. The difference between the two groups was highly significant ($p < 0.005$). See Table (1) for details.

When the intensity of lepromin reaction was compared with the relative density of malaria parasites, and clinical severity of the patients the correlation was not significant. See Table (2) and (3) for details.

Table 1. Lepromin Reaction on falciparum malaria patients and on controls

	Lepromin Reaction	Malaria Patients	Controls
	Neg.	52	6
(Doubtful reaction)	+	15	1
	+	19	4
	++	20	26
	+++	10	63
Total		116	100

Table 2. Reaction between parasite density and lepromin reaction

Lepromin Reaction	Parasite Density				Total
	+	++	+++	++++	
Neg.	11	2	10	29	52
+	5	3	1	6	15
++		2		17	19
+++	3	2	3	12	20
++++	2	1	2	5	10

Table 3. Relation between Clinical Category of malaria patients and Lepromin Reaction

Lepromin Reaction	Clinical Category			Total
	Cat 1	Cat 11	Cat 111	
Neg.	27	22	3	52
+	6	7	2	15
++	3	15	1	19
+++	6	12	2	20
++++	7	2	1	10

DISCUSSION

Lepromin is the crude semistandardised extract of bacilli from a lepromatous nodule. The test is positive in all cases of TT and BT leprosy and invariably negative in BL and LL leprosy.

In this study most of the malaria patients were lepromin negative. Mc Greger and Barr(3) had also found a high incidence of non reactors to tetanus toxoid among children with malaria. Other immune depressive changes in the course of some respiratory and intestinal infections complicated by malaria had also been reported (2). Ho et al. (4) had also reported antigen specific Immunosuppression in human malaria due to *Plasmodium falciparum*. Thus lepromin negative reaction associated with malaria patients were probably due to immunodepression during the active phase of the disease.

ACKNOWLEDGEMENTS

This work was funded by the Institutional Strengthening Grant of UNDP/World Bank/WHO Special programme for Research and Training in Tropical Diseases. We also thank Dr. William Huang for his help.

REFERENCES

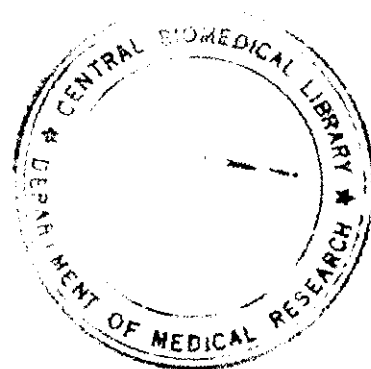
1. Bryceson, A. & Pfaltzgraff, R.E. Leprosy for students of Medicine. Churchill Livingstone. 1973; 62.
2. Bruce-Chwatt. Essential Malariology. Heinemann. 1985; 114.
3. Mc Gregor, I.A., Barr, M. Antibody response to tetanous toxoid inoculation in malarious and non-malarious Gambian children. Trans. Roy. Soc. Trop. Med. & Hyg. 1962; 56: 364-367.
4. May Ho, H. Kyle Webster, Sornchai Loogreesuwan et al. Antigen Specific Immunosuppression in Human Malaria due to Plasmodium falciparum. The Journal of Infectious Diseases. 1986; 153: 763-771.

Accepted for publication 27 Nov. 1990.

MYANMAR
HEALTH
SCIENCES
RESEARCH JOURNAL



Department of Medical Research
Ministry of Health, Yangon, Myanmar



INDEX TO VOLUME 2 (1990)

Author Index

A

Aung-Myo-Han - 135, 138
Aye-Kyaw - 14
Aye-Tha - 107

B

Ba-Thike, K. - 61

C

Cho-Cho-Myint - 47, 111

H

Hamashima, Y. - 50
Hayashi, H. - 118
Hla-Myint - 9
Hla-Pe - 81
Hla-Yee-Yee - 66, 72
Hnin-Hlaing - 114

I

Ikehara, S. - 50

K

Khin-Ei-Han - 77
Khin-Maung-U - 14
Khin-Maung-Win - 9
Khin-Nwe-Oo - 135, 138
Khin-Nyunt - 61
Khin-San-Tint - 61
Khin-Soe-Mu - 84
Kyu-Kyu-Mar - 118

M

MacGregor, G.A. - 66
Mar-Mar-Nyein - 14, 118, 130
Markandu, N.D. - 66
Maung-Maung-Oo - 50
Myat-Lay-Kyin - 19
Myat-Myat-Thu - 91, 152
Myat-Phone-Kyaw - 144
Myint-Oo - 144
Myo-Paing - 32, 39, 44, 88, 91, 96, 102
Myo-Thein - 9

N

Nan-Oo - 61

O

Oku, Y. - 130

P

Pe-Than-Myint - 84, 111, 157
Po, L. - 114

S

San-San-Aye - 14
Saw-Lwin - 61
Sebastian Anthony - 152
Sein-Min - 32, 88, 96
Shirley, D.G. - 66, 72
Singer, D.R.J. - 66
Soe-Soe - 77

T

Tha, J. - 107
Than-Than - 77
Than-Saw - 19
Thein-Hlaing - 1, 19, 25
Thein-Than - 81, 144
Thein-Thein-Myint - 47
Thi-Thi-Naing - 32, 44, 88, 96
Thida-Aung - 9
Tin-Aung - 9
Tin-Aye - 118, 138, 135
Tin-Maung-Maung - 1
Tin-shwe - 47, 84, 111, 157
Tin-Tun - 81
Tin-Tin-Hlaing - 91

Y

Ye-Tint-Lwin - 114

Z

Zaw-Myint - 32, 88, 96
Zin-Zin-Moe - 135

Subject Index

A

Aedes aegypti - 152
Aluminum - 138
Amodiaquine - 84
Anopheles - 32, 39, 44, 91, 96, 102

B

Bacteria - 138
Blood cells - 77

C

Cells, immune response - 50
Chloroquine - 84
Cytotoxins - 118

D

Dengue - 152
Diarrhea - 118
DMR- Larval trap - 152

E

Enterotoxins - 14, 130
enzymes - 14
Escherichia coli - 14, 118, 130

G

glucose - 9
Glucosephosphatase deficiency - 144

H

Hepatitis, viral - 9
Haptoglobins - 81
Helminthiasis - 19
Hypertension - 66

I

Immunity - 50
Insulin - 9

K

Knowledge-perception-behaviour - 1

L

Laparotomy - 25
Lepromin - 157

M

Malaria - 1, 47, 50, 84, 88, 111, 157
Menstrual cycle - 72

O

Ovarian neoplasm - 61

P

Pre-eclampsia - 114

Q

Quinine - 47, 111

S

Snake bites - 81

T

Thalassemia - 144

U

Ultrasonic diagnosis - 61
Uric acid - 114

V

Viper venoms - 77, 107

W

Water microbiology - 135, 138