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Factors associated with HIV seroprevalence among intravenous drug users in Yangon

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A cross-sectional seroprevalence survey was undertaken to determine seroprevalence of HIV infection and the factors associated with HIV seropositivity among the newly registered 205 male intravenous drug users (IVDUs) at the Drug Dependency Treatment and Research Unit in Yangon from December 1991 to September 1992. Of the 178 individuals tested for HIV antibodies, 60.1% were found to be positive, 95% confidence interval (CI) being 51.4 and 68.9%. Moreover, the risk of getting HIV infection was higher among: IVDUs aged 30 years or above than those aged below 25 years [Odds ratio (OR) = 2.76, 95% CI:1.12, 6.81]; IVDUs using drug of over 18 months than those using 6 months or less (OR = 5.07; 95% CI:2.36, 10.87); IVDUs sharing syringes and needles with two or more persons than those who did not share (OR = 2.45; 95% CI:1.02, 5.89); IVDUs visiting to professional drug injectors for initiation of habit than those who did not visit (OR = 2.27; 95% CI:0.96, 5.37); and IVDUs who had given history of imprisonment than those who had not (OR = 2.65; 95% CI:1.04, 6.75). In addition, the above two variables, viz, age and duration of drug use did not confound the effect of needle sharing on HIV infection among the IVDUs. The strengths and weaknesses of the study design and the implications of the study to AIDS control are discussed.

INTRODUCTION

AIDS/HIV infection has become one of the national priority health problem in Myanmar beginning in the fourth National Health Plan for 1990-91 to 1991-92 [1]. As of June 1992, a total of 4313 HIV positive cases were reported in Myanmar [2]. Of the total of HIV positive cases reported, about 65% were found to be intravenous drug users (IVDUs) [3]. Yearly admissions at the Yangon Drug Dependency Treatment and Research Unit (DDT & RU) reached a total of 760 IVDUs in 1989 and 452 in 1990 [4]. Sentinel surveillance carried out on IVDUs at DDT & RU in 1989 and 1990, had shown that the HIV seropositivity rose from 73% in May 1989 to 92% in September 1990 (Ohn Maung, personal communication). Studies on association between needle sharing and other risk behaviours and HIV infection had been undertaken in San Francisco [5], Spain [6] and Italy [7]. As the seroprevalence of HIV positivity among IVDUs in the streets (untreated at DDT & RU) is unknown, and very few or no studies have been done on risk factors among treated and untreated IVDUs in Myanmar, a one-year research project was proposed for the conduct of a cross-sectional study to determine seroprevalence and the possible risk factors for HIV seropositivity not only among the IVDUs treated at DDT & RU but also those in the streets in Yangon. However, just before implementation, the study on IVDUs in the streets had to be abandoned due to unavoidable circumstances. It

was also known that a fairly high proportion of relapsed IVDUs has been admitted to the Unit (Chn Maung, personal communication).

Thus, the objectives of the present communication were confined to the determination of seroprevalence of HIV infection and the comparison of past exposures of interest between the IVDUs with and without HIV infection, who were admitted to the DDT & RU in Yangon for the first time.

MATERIALS AND METHODS

Study subjects:

The sample sizes were determined as follows: (i) For the study of seroprevalence among the newly admitted IVDUs, the number of subjects to be studied was based on the best estimate of 50% prevalence of HIV seropositivity with a confidence level of 95% and allowable error of 10%. This gave the sample size of 96 or about 100 subjects. Assuming that there could be a 20% loss due to various reasons, a sample size of 120 was taken for the seroprevalence study. (ii) For the study of factors particularly needle sharing practices, associated with HIV seropositivity, a case-control methodological approach was undertaken. The number of IVDUs with HIV infection (cases) and without HIV infection (controls) was 104 in number in each group using the formula for determination of sample size for a case-control study of equal case and control size [8]. The calculation was based on the following parameters: the proportion of practising needle sharing with other persons among the controls was 50% (the best guess); confidence level and statistical power were 95% (one-sided) and 80% respectively; and the odds ratio to be tested as being statistically significant was 2.0.

From December 1991 to November 1992, newly admitted drug addicts who were diagnosed as intravenous heroin users by medical officers at the DDT & RU, Yangon, and who came for treatment, were enrolled into the study. Readmitted cases were omitted from the study. Only 205 newly admitted subjects were identifiable during the one-year study period. Of these, 178 subjects were eligible for analysis. 27 cases were omitted due to the following reasons: 11 IVDUs absconded before taking blood; the results of 9 blood samples were unknown due to haemolysis or incomplete testing procedure; 4 IVDUs had previous history of treatment elsewhere; 2 were woman drug users; and 1 user insisted not using IV route and information of drug use was not reliable. There was no incidence of refusal for participation.

Interview:

Each IVDU was interviewed on admission or one to three days after admission depending on the fitness of the user with a standardized questionnaire. All study subjects were requested for informed consent to participate in the study. Interviews were conducted by trained project staff who had not been involved in the care or treatment of the individual. Data were obtained on demographics, drug use and sexual behaviours. Demographic information included age and residential address. Drug use behaviours included history of frequency of needle use, duration of use, needle sharing and cleansing practices and visits to 'professional drug injectors'. A professional drug injector is an unauthorized person who injects drug (heroin) intravenously for money to persons

wishing for initiation and/or maintenance of the habit in a clandestine location. Sexual bahaviours included history of exposure to prostitutes and use of condom during sexual act. History of imprisonment was also taken.

HIV antibody testing:

Since all IVDUs attending the Unit have to undergo test for HIV antibodies, blood samples were collected from all the study subjects. Within 1 to 2 hours after collection, blood samples were transported to National Health Laboratory (NHL). On arrival at NHL sera were separated and stored frozen at -20 degree centigrade until they were being tested. During the test, WHO/GPA testing strategy II [9] was used to detect HIV antibody. All sera were initially tested with indirect ELISA (HIV I ELISA; Schiaparelli Biosystem B.V; Electro-Nucleonics International). Positive serum was retested with the same ELISA. Sera that were negative either on the initial test or on the retest were regarded as HIV antibody negative. Repeatedly positive serum was confirmed by competitive ELISA (Wellcozyme HIV Recombinant; Wellcome Diagnostics). If this supplementary test was found to be negative, it was regarded as HIV antibody negative. On the other hand, if positive, it was regarded as HIV antibody positive. A sample was considered positive only when the presence of antibody was found in the three tests. Using this test system, the sensitivity is about 96% and the specificity is 100% (Soe Lwin, personal communication). The results were recorded in the questionnaire.

Data analysis:

Data entries, analyses and calculations were performed with the software packages, dBASE III plus and EPIINFO VERSION 5. The estimation of seroprevalence for univariate measures was carried out first. Chi-square tests for two-by-two tables and for trends on ordered categorical data were conducted to determine whether there were associations between seroprevalence and each of host factors, drug use and sexual behaviour variables. Being a comparative study between IVDUs with and without HIV seropositivity of their past reported exposures, crude odds ratio (OR) and 95% confidence interval (CI) for each exposure variable were estimated. A simple stratified analysis employing the Mantel-Haenszel adjustment [10] was also done to assess independence of risk factors found significant by univariate analysis, i.e. when the lower bound OR value of 95% CI was greater than 1.0.

RESULTS

Characteristics of study subjects

Among the 178 IVDUs, the median age was 26.0 years and the median duration of drug (heroin) use was 12.0 months. Of the all study subjects, 89.9% were reported to be residing in Yangon city and 10.1% residing outside the city area; 15.2% were reported to have the history of imprisonment with drug-related offenses.

HIV seroprevalence

Of the 178 individuals tested for HIV antibodies, 60.1% were found to be positive, 95% CI being 51.4 and 68.9%.

Factors influencing HIV seroprevalence

Bivariate analysis without adjustment: Table 1 shows the HIV seroprevalence and odds ratio with

Table 1. HIV seroprevalence and odds ratio (95% CI) in relation to demographic characteristics, drug use and sexual behaviours, among newly registered male intravenous drug users

Variable	No. tested	No. positive	% positive	OR (95% CI)	P-value (Trend test/ chi-square)
Age (years)					
<25	74	40	54.1	1.00	
25-29	70	41	58.6	1.20(0.63-2.30)	<0.05
>30	34	26	76.5	2.76(1.12-6.81)	
Residential address (Yangon)					
Suburban	58	34	58.6	1.00	
Periurban	70	43	61.4	1.12(0.55-2.26)	N.S.
Downtown	32	23	71.9	1.80(0.71-4.56)	
Frequency of use per day (times)					
<1	34	17	50.0	1.00	
2-3	111	67	60.4	1.52(0.71-3.27)	N.S.
>4	33	23	69.7	2.30(0.85-6.25)	
Duration of heroin use (months)					
<6	69	28	40.6	1.00	
7-18	51	34	66.7	2.93(1.38-6.22)	<0.001
>18	58	45	77.6	5.07(2.36-10.87)	
Sharing of needle (persons)					
0	28	14	50.0	1.00	
1	74	39	52.7	1.11(0.48-2.56)	<0.025
>2	76	54	71.1	2.45(1.02-5.89)	
Visit to professional drug injectors					
No	139	78	56.1	1.00	
Yes	39	29	74.3	2.27(0.96-5.37)	N.S.
Cleansing of syringes & needles					
Boiling water	24	11	45.8	1.00	
Warm water	50	28	56.0	1.50(0.56-2.67)	<0.05
Cold water	97	65	67.0	2.40(0.98-5.86)	
History of exposure to prostitutes					
No	117	67	57.3	1.00	
Yes	61	40	65.6	1.42(0.75-2.70)	N.S.
History of imprisonment					
No	151	86	56.9	1.00	
Yes	27	21	77.8	2.65(1.04-6.75)	<0.05

95% CI by demographic characteristics, drug use and sexual behaviours, and history of imprisonment among the IVDUs. The increasing trends of HIV seroprevalence by age, duration of IV drug use, number of persons sharing with syringes and needles, and cleansing material were found to be statistically significant. In addition, significant increased risk was found with HIV seropositivity in the former when compared with the latter in the following pairs: IVDUs aged 30 years or above versus IVDUs aged below 25 years (OR = 2.76; 95% CI:1.12, 6.81); subjects using intravenous route more than 18 months versus these using drug less than 6 months (OR = 5.07; 95% CI:2.36, 10.87); IVDUs sharing with two or more persons versus those without sharing with persons (OR = 2.45; 95% CI:1.02, 5.89); and subjects who had history of imprisonment versus those without having the history (OR = 2.65; 95% CI:1.05, 6.75).

Stratified analysis: Based on the findings of significant associations of HIV seropositivity for the variables during the bivariate analysis without adjustment the association between the well defined and important behavioural variable, namely the needle sharing with other persons, and HIV positivity status was adjusted for confounding by age and duration of drug use (Table 2). It was found that the magnitude of excess risk of seropositivity for IVDUs who had shared needles with 2 or more persons in comparison with those who did not share plus who had shared with 1 person was similar before or after adjustment by age or by duration of drug use. The values of OR and 95% CI estimated by Mantel and Haenszel method were exactly the same as those of crude estimates (Table 2), indicating that age and duration of drug use were not confounding factors.

Table 2. Association between needle sharing practice and HIV infection adjusted by age or duration of drug use

Variable for adjustment		No.of persons needle sharing with >2	0+1	Odds ratio	95% Confidence interval
Age(years)					
<26	HIV+	28	28		
	HIV-	15	33	2.20	0.98; 4.92
>27	HIV+	26	25		
	HIV-	7	16	2.38	0.84; 6.74
Total	HIV+	54	53		
	HIV-	22	49	2.27	1.21; 4.27
Duration of drug use (months)					
<8	HIV+	17	15		
	HIV-	14	30	2.43	0.95; 6.23
>9	HIV+	37	38		
	HIV-	8	19	2.31	0.91; 5.85
Total	HIV+	54	53		
	HIV-	22	49	2.27	1.21; 4.27

DISCUSSION

In our present study, seroprevalence of 60% was found to be markedly lower than that for IVDUs attending DDT & RU, conducted during the sentinel surveillance in the previous years. The possible explanation for the observed discripancy between the studies may be due to the fact that subjects in the sentinel report included those old and recurrent cases. In fact, recurrent or readmitted cases comprised 40% of the total IVDUs attending the Unit [4]. Although the study sample was not representative of the entire IVDUs in the community, the selection has not been based on volunteering and there were no cases recorded avoiding the study. The occurrence of very high HIV seroprevalence among IVDUs in comparison with those in other high

risk groups, such as prostitutes (4.32%), males with sexually transmitted diseases (11.37%), females with sexually transmitted diseases (6.42%) that were reported in 1991 [2] indicates that HIV infection has already spread rapidly within the IV drug use community.

As the number of IVDUs in each group of positive or negative HIV serological status was relatively small in comparison with the originally planned sample size, we reassessed the statistical power for the study using the statistical formula for estimation of power for case-control study with unequal case and control size [8]. The following parameters were utilized: significance level of 0.05 (one-sided), proportion of IVDUs with HIV infection (cases) sharing needles with 2 or more persons as 50.5% (54/107) and that of IVDUs without HIV infection (controls) as 31% (22/71) (from Table 2). The power was found to be over 80% which was the value that we have originally planned. As in other studies carried out in San Francisco [5], Spain [6] and Italy [7], regular sharing of needles with other persons among IVDUs in Yangon were significantly associated with HIV seropositivity even after removing the effect of age or duration of drug use by stratified analysis.

As IVDUs visiting professional drug injectors for initiation of the habit had 2.3 times of getting the infection than those who did not, the role played by these professional injectors in the transmission of HIV infection should not be neglected. The other variable that had a significant correlation with HIV positivity was history of imprisonment. The imprisonment may be related to intravenous heroin use.

In this study, sexual mode of transmission might not have been an important mode of transmission among IVDUs, although a recent Italian study [11] has shown that sexual transmission played a relevant and additive role in HIV infection among IVDUs who had sexual partners with HIV infection (OR = 4.8; 95% CI:1.9, 12.0).

The study has the following limitations: (i) detailed history of drug use behaviour such as the number of persons sharing with needles in connection with professional injectors, and before imprisonment, and of sexual behaviour such as number and rate of change of sexual partners in connection with exposure to prostitutes; (ii) validation of data had not been carried out by performing two or more independent measurements on the same exposure variable and comparing the findings; (iii) the data analysis was based on seroprevalence data and therefore the time of HIV seroconversion could not be known; and (iv) the misclassification error especially non-differential one, might have been present in relation to needle sharing practices.

However, there are many strong points in the study. (i) The study was confined to male intravenous heroin users only. (ii) The interviewers were well trained and the questionnaire used was standardized to increase the reliability and thus validity of the data. (iii) The history of exposure was taken from IVDUs before their seropositivity status was known and therefore, even though non-differential misclassification error occurred, the direction of the magnitude of odds ratio would be towards the null, i.e. the actual odds ratio would in fact be higher than the present estimate. (iv) The findings

on association between needle sharing practices and HIV infection in this study are similar to those of other corss-sectional studies [5,12,13] and a prospective study [11]. Hence, given the above limitations, this study has the following implications for AIDS control: (i) IVDUs without needle sharing has the least risk and sharing with 2 or more persons has the greatest risk of acquiring HIV infections among IVDUs; (ii) Visiting to professional injectors for initiation of drug use is a possible cause of acquiring the infection probably through needle sharing; and (iii) an appropriate material is required for cleansing syringes and needles to minimize the acquisition of infection.

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A knowledge-attitudes-practices study on AIDS/HIV infection in intravenous drug users at Yangon Drug Dependency Treatment and Research Unit

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A standardized questionnaire was administered to 198 new and 112 recurrent cases of intravenous drug users enrolled at the Drug Dependency Treatment and Research Unit in Yangon to elicit knowledge about, attitudes towards and practices on AIDS/HIV infection. The respondents were all males, with the mean age of 26 years among new and 28 years among recurrent cases. Over 90% of both groups resided in Yangon City. Recurrent users had higher level of knowledge about correct mode of transmission of HIV infection than new cases. For instance, over 73% of recurrent and about 50% of new cases knew that HIV infection could not be transmitted via air or drinking water. 93-97% of recurrent patients believed that transmission of HIV infection was possible through blood, sexual contact or IV drug use against 85-92% of new cases. Concerning drug using and sexual behaviours, recurrent cases took more frequent injections per day, prefer.ed to be alone during drug use, did not take more care in cleansing syringes and needles, but had lesser contact with sex workers than new ones. The findings are compared with those of similar studies in other risk groups. The implications of the study are discussed.

INTRODUCTION

AIDS has emerged as a worldwide public health problem. In Myanmar, the cumulative number of HIV positive cases had reached 4313 as of June 1992 [1]. A series of knowledge-attitudes-practices (KAP) studies have been conducted on high and low risk groups of individual such as sex workers [2], medical [3] and para-medical personnel [4], married couples [5] since 1991. However, there is still a lack of KAP studies among IVDUs in Myanmar. In fact, IVDUs are at high risk category of individuals for spreading of the infection from one another through needle sharing practices as well as to their spouses through sex. Thus, a study was undertaken on a group of IVDUs to assess the level of knowledge about and attitudes towards AIDS/HIV infection, and practices on prevention of the infection in Yangon.

MATERIALS AND METHODS

Study subjects: From December 1991 to November 1992, newly admitted IVDU patients attending Drug Dependency Treatment and Research Unit (DDT & RU), Yangon, were enrolled in the study. Patients with the history of intravenous drug use were eligible. There were missed cases during the study period due to the patients who absconded between one to three days after admission. At the end, a total of 205 study subjects were examined and

there were no incident of refusal for participation. Of these, 198 study subjects were eligible for data analysis. The others (2 woman drug users, 4 users with previous history of treatment elsewhere and 1 user who insisted not using IV route) were omitted for the interview. In addition, 112 old IVDUs who had already admitted at least once at the DDT & RU were included in the study.

Interview: During the preparatory phase questionnaires were developed by interviewing and reinterviewing hospitalized IVDUs. After pretesting the questionnaires, the interview was conducted by trained technicians. Each intravenous drug user was interviewed at admission, or one to three days after admission depending on the fitness of the user with the standardized questionnaires. All study subjects were requested to consent to participate in the study. The trained project staff were not involved in the care or treatment of the individual.

Data analysis: Comparison between new and old IVDUs was made with respect to characteristics of respondents, proportion of IVDUs with little knowledge about AIDS, misconception about AIDS and behaviours favouring transmission of the infection. Chi-square test was used to show any significant differences.

RESULTS

Demographic variables

Regarding age-group distribution of the respondents, about 40% were at the age group of 25-29 both in old and new IVDUs. The mean age was 26.0 years for new and 28.0 years for old IVDUs. About 90% of IVDUs old and new were living in Yangon and only few i.e. 4-10% were from districts (Table 1). Among IVDUs (old & new), it was noted that 15% of IVDUs had been jailed.

Table 1. Percentage distribution of characteristic of respondents among intravenous drug users (IVDUs)

Characteristic	New IVDUs (n = 198)	Old IVDUs (n = 112)
Age (years)		
<20	8.1	3.6
20-24	33.3	25.0
25-29	39.9	37.5
>=30	18.7	33.9
Mean	26.45	28.98
	X^2= 9.29; p<0.02	
Residence		
Downtown, Yangon	19.7	30.4
Suburban, Yangon	31.8	33.9
Periurban, Yangon	38.4	32.1
District	10.1	3.6
	X^2= 6.81; p<0.05	

Knowledge on AIDS/HIV infection

99% of the new and 100% of the old subjects have ever heard of AIDS. Regarding transmission of the disease, 54% of the new and 76% of the old knew correctly about not getting AIDS through air; 48.5% of the new and 73% of the old IVDUs not getting HIV infection through water; 88% of the new and 97% of the old getting the infection through infected blood; 92% of the new and 97% of the old cases getting HIV infection through sex; and 86% of the new and 94% of the old about getting the infection by using drugs intravenously (Table 2).

Attitudes towards AIDS

88.8% of the new and 85.7% of the old cases disagreed that AIDS is a

Table 2. Percentage distribution of knowledge variable about AIDS/HIV among intravenous drug user (IVDUs)

Knowledge variable	New IVDUs (n = 198)	Old IVDUs (n = 112)
Have you ever heard of AIDS?		
Yes	98.8	100.0
No	1.2	
	X^2= 0.11; p>0.5	
Getting HIV infection via air		
Yes	13.8	
Don't know	32.1	24.1
No	54.1	75.9
	X^2= 20.33; p<0.001	
Getting HIV infection via drinking water		
Yes	25.5	9.8
Don't know	26.0	17.0
No	48.5	73.2
	X^2= 17.44; p<0.001	
Getting HIV infection via blood		
Yes	88.3	97.3
Don't know	10.7	2.7
No	1.0	
	X^2= 5.57; p>0.05	
Getting HIV infection via sex		
Yes	92.3	97.3
Don't know	5.6	1.8
No	2.0	0.9
	X^2= 1.87; p>0.10	
Getting HIV infection IV drug use		
Yes	86.2	93.8
Don't know	12.2	3.6
No	1.5	2.7
	X^2= 5.43;p>0.05	

curable disease. 86.7% of the new and 93.8% of the old agreed that they are at risk of getting AIDS (Table 3).

Drug-use practices and sexual behaviours

Table 4 shows the drug-use behaviours among IVDUs. 60.6% of the new and 77.7% of the old cases were less than 25 years of age at first use. 61.1% of the new and 67% of the old used the drug 2-3 times a day. Total duration of drug used within 6 months or less occurred among 39.4% of the new and among 76.8% of the old cases. Most of IVDUs shared needles with one partner or more constituting at 84.3% among the new and 68.9% among the old cases. For cleansing of needles and syringes, 58.6% of the new and 76.8% of the old used cold water. Some 27% of the new and 16% of the old cleaned with warm water. Very few (13.6% of the new and 5.4% of the old cases) used boiled water for cleansing their equipment. 39.8% of the new and 21.4% of the old cases had history of contact with sex workers. However, only 44.9% of the new and 20.8% of the old IVDUs used condoms.

Table 3. Percengage distribution of attitude variable towards AIDS among intravenous drug users (IVDUs)

Attitude variable	New IVDUs (n = 196)	Old IVDUs (n = 112)
Do you think AIDS can be cured?		
Agree	4.6	5.4
Don't know	6.6	8.9
Disagree	88.8	85.7
	X^2= 0.29; p>0.5	
Do you think IVDUs are at risk of getting AIDS ?		
Agree	86.7	93.8
Don't know	9.2	2.7
Disagree	4.1	3.5
	X^2= 3.85; p>0.10	

Table 4. Percentage distribution of drug-use behaviour among intravenous drug users (IVDUs)

Variable	New IVDUs (n = 198)	Old IVDUs (n = 112)
Age of first use		
<20 years	28.8	37.5
20-24	31.8	40.2
25-29	28.8	14.3
30+	10.6	8.0
	X^2= 8.69; p<0.02	
Frequency of use		
<1 time/day	19.2	7.1
2-3	61.1	67.0
>3	19.7	25.9
	X^2 = 7.5; p<0.05	
Duration of drug use		
<6 months	39.4	76.8
7-18	28.3	12.5
>= 19	32.3	10.7
	X^2= 38.17; p<0.001	
No. of persons sharing needles		
Alone	16.7	41.1
With 1 partner	41.4	32.1
With >= 2	42.9	36.8
	X^2= 21.24; p<0.001	
Duration of visiting professional injector		
0 months	79.8	Not
<30	11.1	asked
>= 30	9.1	
Use of cleansing materials		
Cold water	58.6	76.8
Warm water	27.2	16.0
Boiling water	13.6	5.4
Other	0.6	1.8
	X^2= 10.65; p<0.01	

DISCUSSION

Although 99% of new and 100% old cases had ever heard of the disease, a number of misconceptions existed. For instance, some 13.8% of the new IVDUs stated that HIV could be infected through air. A group of new (25.5%) and old (9.8%) thought that HIV infection could be transmitted through water. However, the level of knowledge of HIV infection/AIDS among IVDUs is quite satisfactory when compared with other risk groups like married couples, paramedical personnel.

The development of effective strategies to prevent HIV infection requires an understanding of the behaviours that place individuals at risk and of those factors that are related to behaviour change. One of the major factors in transmission of the diseases is needle sharing practices. In this study, the majority had history of needle sharing with at least one partner. In addition, visiting to professional injector is an important key issue as they used to inject the same needle for several drug users. The average duration of visit to professional injector was found to be about 2 years among the new cases. It means that there has been a cycle of transmission among partners of needle sharing, and some of them may not attend the treatment centre. It is thus felt that the hidden IVDUs are still left in numbers to be found out. The use of cleansing materials for their syringes and needles are also not satisfactory. Besides, one third of IVDUs had history of contact with sex workers and only a few used condoms during sexual relationship. Thus, there was a gap between knowledge and practices on prevention of AIDS/HIV infection in this study.

It is felt that specific measures should be considered to prevent the spread of HIV infection among IVDUs as well as from them to their families and to the whole community. Thus, the efforts should be placed for AIDS prevention programme not only on health education dissemina-

tion but also, on provision of effective counselling services, partner notification, promotion of safe sex practices.

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Blood sugar lowering effect of Momordica charantia L. fruit (Kyethingha thee) in rabbit model

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In Myanmar, diabetes mellitus can be counted as a single health problem occuring at all ages as reported by various workers. Momordica charantia L. fruit (Kyethingha thee) has been reported to possess anti-diabetic activity in experimental animal model by various workers. The plant is widely distributed and also cultivated in Myanmar for its edible fruits. Therefore, it was thought that, if it will be worthwhile to investigate and confirm experimentally, whether the fruit grown in Myanmar possess similar hypoglycemic activity. Blood glucose levels of adrenaline-induced diabetic rabbits were determined after oral administration of expressed fruit juice (10 ml/kg) which was approximately equivalent to 400 mg/kg of the substance significantly inhibited the hyperglycemic blood glucose level on adrenaline-diabetic rabbits at 2 hr.

INTRODUCTION

In Myanmar, diabetes mellitus can be counted as a single health problem occuring at all ages as reported by various workers. Many of the plants had been claimed in relieving diabetes mellitus, and even attaining radical cure. Mya Bwin and Sein Gwan [1] and Oliver [2] mentioned a large number of herbal drugs which are reputed for the treatment of diabetes mellitus. Farnsworth [3] stated that "One of the prerequisite for the success of primary health care is the availability and use of suitable drugs. Plants have always been a common source of medicaments either in the form of traditional medicine formulations or as pure active principles".

Myanmar is rich in plants many of which are of medicinal values. But, so far, there has not been full and systematic exploitation of these natural resources with regard to hypoglycaemic effect. So, it is rational to screen the plant extracts preliminarily on laboratory test models. As Momordica charantia Linn. fruit (Kyethingha thee) has been reported to possess anti-diabetic activity in experimental animal model by various workers, it was thought that, if it will be worthwhile to investigate and confirm experimentally, whether the fruit grown in Myanmar possess similar hypoglycaemic activity.

Kyethingha, commonly known as "Bitter gourd" is a climbing or spreading shrub, with alternately arranged, five to seven deeply divided rounded sinuses with sharp-pointed notched lobed, roundish, cordate base leaves, and bright orange ovoid fruit bearing numerous triangular tubercules. Its flowering and fruiting period is during the rainy months. It is widely distributed and cultivated in Myanmar Naingngan for its edible fruits.

MATERIALS AND METHODS

(a) Preparation of the fruit juice and watery extract

The fruits used for the experiment were purchased from the markets of Yangon area. The fresh fruits were weighed, sliced into small pieces and homogenized in a homogenizer (Polytron Homogenizer, Kinematica, Switzerland). The homogenized material was squeezed through cheese cloth to remove fabric materials. The expressed fruit juice was partitioned into solid and liquid layers by means of centrifuge (IEC International Centrifuge, International Equipment Company, U.S.A.). Thus the turbid supernatant was decanted and pooled in a 500 ml conical flask.

Crude watery extracts were prepared by refluxing 50 g of air-dried fruit powder in a round bottomed flask on a boiling water bath using 500 ml of distilled water for about 6 hrs. It was filtered using cheese cloth and the filtrate was evaporated to dryness on a boiling water bath. The aqueous extract obtained was dissolved in 1% methyl cellulose before administration to respective animals.

(b) Qualitative test for the fresh fruit juice and the watery extract

Qualitative identification tests of the chemical constituents present in the tested samples were conducted according to the standard procedure as stated in Trease and Evans [4] and Pharmacopaeia [5].

(c) Inhibitory effect of fruit juice and water soluble substance on adrenaline-induced hyperglycaemia in rabbits

Adult healthy rabbits of JB strain weighing 2.6 ± 0.20 kg were used in these experiments. The rabbits were made diabetic by injecting them subcutaneously with 0.1 mg/kg of adrenaline tartrate injection using the method of Gupta, Verma, Garg and Mahesh Rai [6]. This study was conducted on rabbits which had been divided into 4 groups of 3 rabbits each. Group I (Control) was given 1% methyl cellulose as the placebo; Group II, the expressed fruit juice (10 ml/kg at 0h and 1h); Group III, the standard drug, glibenclamide (8 mg/kg) and Group IV, the aqueous extract (3 g/kg) respectively. Fasting blood samples were collected at 0h after which adrenaline tartrate was immediately administered subcutaneously to all the rabbits following the oral administration. The serial blood samples were again collected at 1h, 2h and 4h respectively for estimation of blood sugar levels which were determined by the O-toluidine method [7]. Cross over tests were done after a week's interval so that all the rabbits remained as control at one time and they had treatment with other test drugs at another time.

(d) Test for fresh fruit juice on oral glucose tolerance test (OGTT) in rabbits

The glucose tolerance test on rabbit was performed as the above stated method. The only difference was the first 3 rabbits was given glucose (3 g/kg) only and the second group was given glucose (3 g/kg) dissolved in expressed fruit juice (10 ml/kg) according to the method stated by Mukherjee, Ghosh and Datta [8]. For a time course of peak interaction, 1h, 2h and 4h later, blood samples were taken from each individual animal and the blood sugar levels were estimated. Cross over tests were done as the previous test.

RESULTS

(a) Qualitative test for the fruit juice and watery extract

It was found that (100 g) of fruit produced (40-50) ml of liquid juice, after centrifuging and soluble solid in this juice was approximately 4 g/100 ml. The yield of water soluble extracts from dried powder of fruit was 28 g/100 g. The results from the qualitative identification tests on the fresh fruit juice and water soluble extracts were shown in Table 1.

Table 1. Qualitative tests of fruit juice and water soluble extracts of Momordica charantia L. (Kyethingha thee)

Samples	Alkaloids	Flavonoids	Amino acid	Tanninoids	Steroids	Triterpene	Saponins	Reducing sugar	Glycoside	Chloride	Sulphate	Nitrate	Phosphate
Fruit juice	+	-	+	-	-	+	-	+	-	+	-	+	-
Water soluble extract	-	-	+	-	-	+	-	+	+	-	-	+	-

(b) Effect of fruit juice, watery extract and glibenclamide on glucose levels in diabetic rabbits

The mean blood glucose of control and treated animals after oral administration of fruit juice, watery extract and glibenclamide at various time intervals were shown in Fig. 1. These figures showed that the injection of adrenaline significantly induced hyperglycaemia ($p<0.005$) in the rabbits reaching its maximum in 1-2 h. Administration of 10 ml/kg of fruit juice produced a significant inhibition ($p<0.05$) in blood glucose at 2h period when compared with that of the control group. But rabbits treated with 3.0 g/kg of dry fruit watery extract which was equivalent to 12 g/kg dry fruit powder did not show blood sugar levels inhibition throughout the 4h period. The standard test-agent glibenclamide significantly inhibited the blood glucose level when comparison was made between the test and control group at 2h ($p<0.05$) and at 4 h ($p<0.025$).

(c) Effect of fresh fruit juice on glucose levels in glucose loaded rabbits

The mean blood glucose control and treated animals after oral administration of fruit juice at various time intervals were shown in Fig. 2. These figures showed that the administration of glucose significantly induced hyperglycaemia ($p<0.005$) reaching its maximum at 2 h. In the case of animal which received fruit juice along with the sugar, no such rise in blood sugar level at 2 h and 4 h respectively. There was a significant inhibition of hyperglycaemia at 2 h ($p<0.05$) and at 4 h ($p<0.025$).

DISCUSSION

A preliminary dose finding study of the fresh fruit juice on the adrenaline-induced model indicated that a dose of 10 ml/kg (400 mg/kg)

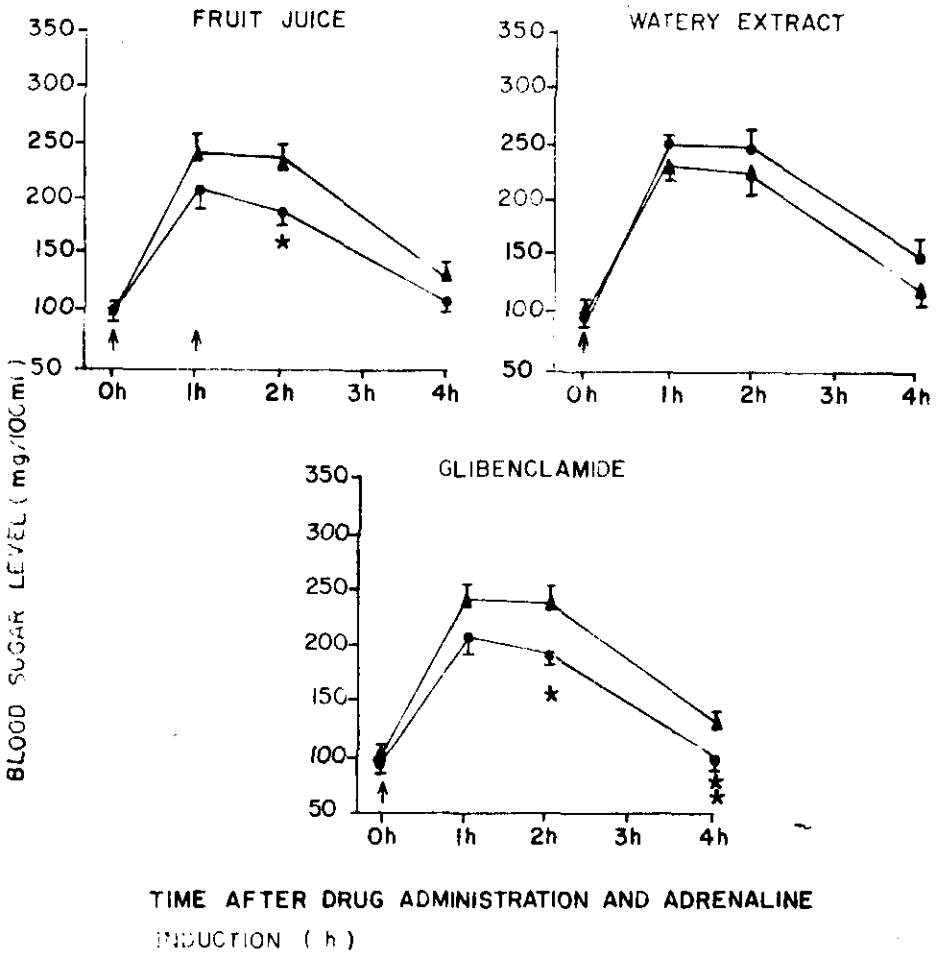

Fig. 1. Time course of the effects of M. charantia fruit juice, its watery extract and *glibenclamide on adrenaline-induced hyperglycaemia* rabbit model.

Each point represents the mean of observations and veritcal bars indicate standard errors of the means. The groups tested were; ▼——▼ , placebo and ●——● , the test agents respectively. ↑ arrow shows the point for oral administration of drug.

*** denotes $p<0.05$**

**** denotes $p<0.025$**

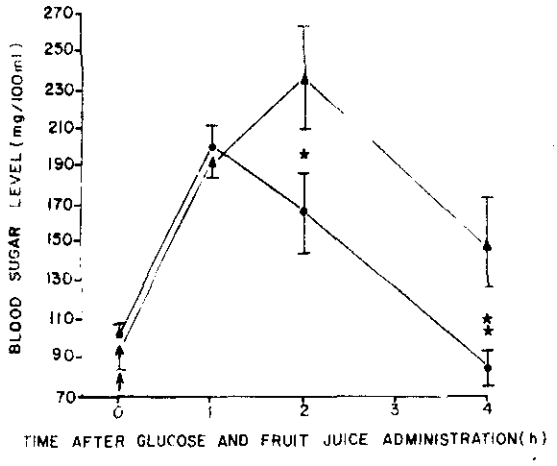

fig. 2. Time course of the effect of M. charantia fruit juice on glucose loaded hyperglycaemia rabbit model

Each point represents the mean of observations and vertical bars indicate standard errors of the means. The groups tested were; v—v, placebo and o—o , the test fruit juice respectively. ↑ arrow shows the point for oral administration of glucose and fruit juice.

*** denotes $p<0.05$**

**** denotes $p<0.025$**

which was the maximum practical amount that could be administered to a rabbit model, showed a decreasing trend in blood glucose level. But the effect was not statistically significant. A double dose of the above dose which was 20 ml/kg (800 mg/kg) was then tried out, but since the bulk of the juice was more than that which the animal could tolerate, the dose was administered in two divided doses at 1 hour interval (i.e. 10 ml/kg or 400 mg/kg at a time). In this investigation, the juice caused a significant inhibition ($p<0.05$) of the hyperglycaemic response to the induction of adrenaline. Similarly, glibenclamide (8 mg/kg) caused a significant inhibition ($p<0.025$) of the hyperglycaemic response. With the fruit juice, the peak of action was found to be at 2 h. But the aqueous extract (3 g/kg) did not produce inhibition of the hyperglycaemic response to the induction of adrenaline. There was also a significant inhibition of the fresh fruit juice in blood sugar level at 2 h and 4 h in Oral Glucose Tolerance Test (OGTT).

So it can be concluded that fresh fruit juice possesses an active principle with definite decrease of blood sugar level. Presence of alkaloid like material was also detected in fresh juice of Kyethingha thee. Karunanayake et al. [9] stated the effect of Kyethingha thee juice on streptozotocin-induced diabetes in rats. Chatterjee [10] stated that the fruit contains antidiabetic principle and the centrifuged juice was more active than the alcoholic extract. Diaz [11] reported that powdered drug of the plant contains alkaloid.

Singh et al. [12] also reported the effect of long term feeding of acetone extract of whole fruit powder on alloxan diabetic albino rats. Rivera [13] and Welihinda et al. [14] mentioned that there is some hypoglycaemic action on the sugar tolerance of normal rabbits.

Lotlikar and Rao [15] stated the beneficial clinical results reported from using daily 50-60 ml of the juice of fresh fruits. Akhtar [16] reported the trial of powder in patients with maturity onset diabetes. Day et al. [17] also stated the presence of at least two types of orally active hypoglycaemic principles in Kyethingha thee a more rapidly effective water soluble component, and a more slowly acting component possibly an alkaloid. Akhtar et al. [16] hypothesized that the Kyethingha thee containe more than one type of hypoglycaemic

components which may include an alkaloid and an orally active insulin-like compounds. According to our chemical investigation, the significant difference between the juice and watery extract is the presence of alkaloid in the juice. Therefore, further chemical investigation needs to be pursued regarding the active principle.

Hyperglycaemia due to adrenaline was known to be effected partly through release of adreno-corticoids as stated by Gupta et al. [18] and partly through stimulation of the adreno-hypoglyceal axis as stated by Voght [19]. Thus, the action of the fruit juice may probably be effective through either direct glycogenolysis or indirectly through action on the adrenocortico-hypoglyceal axis by inhibiting the gluconeogenesis. Further comprehensive chemical and pharmacological investigations are needed to elucidate the exact mechanism of hypoglycaemic effect of Kyethingha thee.

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Screening of some medicinal plants reputed for anthelminthic activity on *in vitro* test models

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Thirty three medicinal plants, traditionally claimed to he useful for purging human intestinal roundworms were evaluated experimentally for their anthelminthic action against *Ascaris summ in vitro*. On the basis of producing muscle paralysis of the worm within predetermined experimental periods, seventeen of the plants tested were found to possess anthelminthic activity. *Urginea indica* and *Ananas sativa* were most effective and a fungus, *Hydnum repanda* also showed good activity.

INTRODUCTION

In Myanmar, ascariasis can be counted as a single health problem. Various workers reported that the wet and humid rural areas of Myanmar are endemic to intestinal helminthiasis [1,2] particularly to ascariasis [3]. The predominant intestinal helminth is *Ascaris lumbricoides* as observed in previous surveys [1,2] and the study in Yangon [4]. Many authors also reviewed the national experiences of ascariasis control measures and programmes [5,6]. It was also reported that *Ascaris lumbricoides* was the principle intestinal helminth and its infection rate was 77 percent in the population as a whole [7].

Myanmar is rich in plants many of which are of medicinal values. But, so far, there has not been full and systematic exploitation of these natural resources with regard to ascariasis. So, it is rational to screen 33 plant extracts, traditionally claimed to be useful for purging human intestinal round worms and not yet scientifically reported, were evaluated for anthelminthic activity against *Ascaris suum*.

MATERIALS AND METHODS

(a) Preparation of plant extracts

The 33 plants to be tested were collected from various parts of Myanmar. Plants for screening were botanically identified according to [8,9] by one of the authors who is a botanist and specimens representing the collection were deposited in the herbarium of the Pharmacognosy Laboratory, Pharmacology Research Division, Department of Medical Research, Yangon. 500 gm of each air dried powder (parts of plants used are detailed in Table 1) were refluxed in a 2 litre round bottomed flask on a boiling water bath using 1 litre of the solvents specified in Table 1. After 12 hours the extracts were filtered and allowed to cool. Filtrates were concentrated to dryness under reduced pressure. The extracts were dessicated for 2 days and the dry residues thus obtained were dissolved in Tyrode's solution and

tested for in vitro anthelminthic activity. Fresh juices of some plants were prepared by using a homogenizer and filtered through cheese cloth.

(b) General anthelminthic screening

Plant extracts were screened by a modification of the method of Sen and Hawking [10]. Experiments were conducted using adult Ascaris suum of either sex collected freshly from slaughtered pigs. These were bathed in Tyrode's solution during transport to the laboratory where they were then incubated at 37°C for 1-2 hr in wide-mouth cylindrical, amber-coloured bottles of 250 ml capacity. The most active worms of both either sex were selected and used for test. Individual plant extracts were dissolved in modified Tyrode's solution and the pH was adjusted to 7.4. For each plant extract tested a total of 10 worms were used. Two worms were put into each of 5 vessels containing 100 ml of the extract preparation and were observed at timed intervals. For the control group, modified Tyrode's solution alone was used as the incubation medium at the temperature of 37°C. The movement of the worms in incubation medium increased on exposure to 60 watt bright light or when touched with a glass rod. These two stimuli were used to determine paralysis in doubtful cases. Paralysed worms which did not move even after 1 min exposure to light or by placing them into warm (about 37-40°C) Tyrode's solution, and which did not respond to the prick stimulus were considered as dead.

(c) Specific screening on the neuromuscular apparatus of Ascaris suum

The most potent extracts of A. sativa, U. indica and H. repanda were tested for anthelminthic activity using the method of Goodwin [11] as modified by Thawka-Kyin [12]. Each selected worm was subjected to a "stocking" process and mounted for recording. The cotton thread was attached to a Myograph Transducer (A-188, E & M Instrument Company, Inc., Houston, Texas, U.S.A.), which was directly connected to the amplifier of a four channel direct writing ink recorder (Physiograph, E & M). Motility measurements of frequency and magnitude were made both before and after the addition of test extract into the Tyrode's solution. Recordings of 15 minutes duration were made every hour throughout each experiment. The motility frequency of each individual worm was calculated from the values obtained at each 15 minutes recording session, and expressed as a percentage of its own "control" value recorded before exposure to test extract. Similarly, the motility magnitude of each individual worm was calculated from the values obtained at each 15 minutes recording session (taken as the average of the contraction heights recorded over each 15 minutes period in mm), and was expressed as a percentage of its own control recorded before exposure to test extract. At least half an hour was allowed for each worm to settle down before exposure to extract. Concentrations of the test extracts employed were 6, 16 and 40 mg/ml final bath concentration in modified Tyrode's solution. A continuous recording was taken for one hour after adding the drug. Then the tracings were recorded for 15 minutes period every hour, up to 6 hours. Thus, extracts remained in contact with the worm for the whole period of observation. A control test was concurrently performed using only Tyrode's solution. The results were compiled and comparative efficacies of the most active plant extracts with respect to the

control was performed statistically using the unpaired Student's 't' test.

RESULTS

(a) General Screening

List of plants on the in vitro anhelminthic activity of the extracts of 32 plants and 1 fungus are shown in Table 1. Of the extracts screened A. sativa, U. indica and H. repanda were the more potent where the test worms were dead within 24 hours; the remaining 8 plants are moderately active where the viability of the parasite was 24-48 hours; and the remaining 6 plants were fairly active where the drug effect was obtained in 48-72 hours respectively (Table 2) under the experimental condition as described by Kaleysa [13]. The rest of the plants listed in Table 1 were less active where the drug effect was obtained in more than 72 hours. Thus, comprehensive anthelminthic efficacy of the plant extracts were finally assessed and expressed as the time taken for 100% lethalities of the worms.

Table 2. Ascariscidal effects of selected plant extracts on the viability of A. suum on in vitro model

List of plants in scientific name	100% lethal concentration(mg/ml)	Duration of viability of the worms in hrs.
1. A. sativa	40	16.8± 0.05*
2. U. indica	6	21.6± 1.20*
3. H. repanda	16	21.6± 0.72*
4. N. arbortritis	32	28.8± 2.69*
5. W. tomentosa	32	31.2± 1.44*
6. C. album	32	33.6± 1.92*
7. C. flaribunda	32	34.8± 0.96*
8. E. robusta	32	36.0± 1.68*
9. C. tora	32	36.0± 1.44*
10. C. pilosum	32	36.0± 0.96*
11. C. auriculata	32	43.2± 0.96*
12. C. burmanni	32	54.0± 4.56*
13. M. phillipinesis	32	55.2± 4.80*
14. S. grandiflora	32	55.2± 3.84*
15. M. azadarach	32	60.0± 2.88*
16. L. glauca	32	69.6± 9.60
17. A. calamus (leaf)	32	69.6±12.24*
Control	0	117.6± 4.60

*** = significantly different when compared with that of the control at p<0.005**

(b) Specific Screening

The effect of the three test agents on the specific test model is shown in Fig. 1 and Fig. 2. The most active plant was A. sativa which killed the worms within 24 hrs at a concentration of 40 mg/ml. The worm became paralysed after about 6 hr and complete inhibition was observed after 18 hrs. No significant stimulation effect of the drug on the movement of the worm was observed. But in the case of U. indica there occured apparent

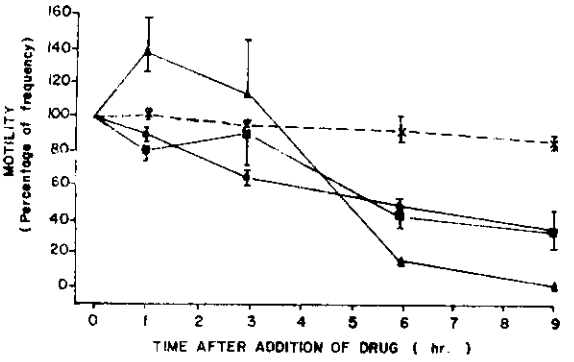

Fig. 1. Time-course of the effects of the plant extracts on the percentage rate of contraction of Ascaris suum

The motility of the parasite is expressed in terms of percentage frequency of its own control per 15 min. Each point represents the mean of observations and vertical bars indicate standard errors of the means. The concentrations and number of observations of the extracts used are A. sativa, 40 mg/ml (n = 6; —■—); U. indica, 6 mg/ml (n = 6;—▼—); H. repanda, 16 mg/ml (n = 5, —○—) and control (n = 6, —●—).

Table 1. List of plants tested for anthelminthic activity against Ascaris suum

	Scientific name	Family	Part used	Extract tested	100% lethal concentration mg/ml
1.	Memordica charantia Linn.	Cucurbitaceae	fruit	50% alcoholic extract	32
2.	Luffa aegyptiaca Mill.	Cucurbitaceae	seed	"	32
3.	Chenopodium album Linn.	Chenopodiaceae	leaf	"	32
4.	Cleodendrum indicum (L.) O.Ktze	Verbenaceae	leaf	"	32
5.	Combretum pilosum Roxb.	Combretaceae	leaf	"	32
6.	Nyctanthes arbortristis Linn.	Oleaceae	leaf	"	32
7.	Sesbania grandiflora Pers.	Papilionaceae	leaf	"	32
8.	Tephrosia purpurea Pers.	Papilionaceae	leaf	"	32
9.	Tinospora cordifolia Miers.	Menispermaceae	stem	"	32
10.	Ficus religiosa Linn.	Moraceae	bark	"	32
11.	Costus speciosus Smith.	Zingiberaceae	rhizome	"	32
12.	Dioscorea sativa Linn.	Dioscoreaceae	rhizome	"	32
13.	Urginea indica Kunth.	Liliaceae	bulb	"	6
14.	Hydnum repanda*		whole plant	95% alcoholic extracts	16
15.	Embelia robusta Burm.	Myrsinaceae	seed	"	32
16.	Caesalpinia crista Linn.	Caesalpiniaceae	leaf	"	32
17.	Calycopteris floribunda Lam.	Combretaceae	leaf	"	32
18.	Cassia auriculata Linn.	Caesalpiniaceae	leaf	"	32
19.	Mallotus philippenensis Muell Arg.	Euphorbiaceae	leaf	"	32
20.	Wrightia tomentosa Roem sch.	Apocyanaceae	leaf	"	32
21.	Leucaena glauca Benth.	Mimosaceae	seed	watery extract	32
22.	Aegle marmeles Corr.	Rutaceae	whole plant	"	32
23.	Artemisia pallens DE.	Compositae	"	"	32
24.	Artemisia vulgaris Linn.	Compositae	"	"	32
25.	Barringtonia acutangula Gaerth.	Myrtaceae	"	"	32
26.	Cassia tora Linn.	Caesalpiniaceae	"	"	32
27.	Cleome burmanni W. & A.	Capparidaceae	"	"	32
28.	Grewia microcos Linn.	Tiliaceae	"	"	32
29.	Melia azadarach Linn.	Meliaceae	"	"	32
30.	Michelia champaca Linn.	Magnoliaceae	"	"	32
31.	Mollugo spergula Linn.	Ficoidaceae	"	"	32
32.	Ananas sativa Linn.	Bromeliaceae	fruit	fresh juice	40
33.	Acorus calamus Linn.	Araceae	leaf	"	32
	"	"	rhizome	"	32

* Fungus

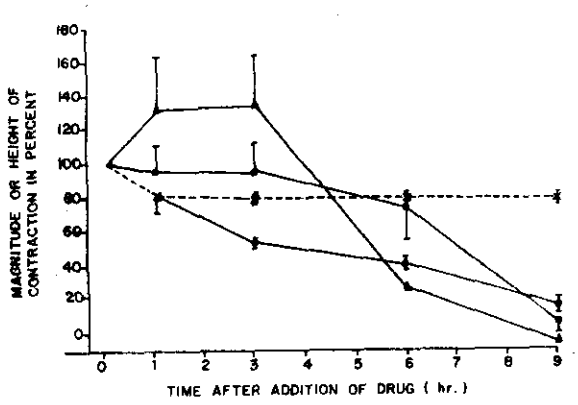

Fig. 2. Time-course of the effects of the extracts on the magnitude or percentage height of contraction of Ascaris suum

The magnitude of the parasite is expressed in terms of percentage height of contraction of its own control per 15 min. Each point represents the mean of observations and vertical bars indicate standard errors of the means. The concentrations and number of observations of the extracts used are A. sativa, 40 mg/ml (n = 6; —●—); U. indica, 6 mg/ml (n = 6; —▲—); H. repanda, 16 mg/ml (n = 5; —○—) and control (n = 6; —×—).

stimulation of parasite activity resulting in both the increase in frequency and the heightening of the magnitude when compared with that of the control. However, mobility of the worm reduced later on resulting in paralysis with significant immobilization in 4-6 hr for both the frequency ($p<0.005$) and magnitude ($p<0.025$), according to Student's 't' test. The test parasite never recovered ultimately leading to death. Regarding the effective fungus H. repanda the effective dosage was found to be 16 mg/ml. The worm became paralyzed after 3 hrs and complete inhibition was observed within 24 hrs experimental period.

DISCUSSION

Thirty-three plants reported to possess anthelminthic activity in literature like Ashin Nagathein [8] and Chopra and coworkers [9] were hereby evaluated. Out of 33 plants tested 17 plants were found to be effective against A. suum in vitro. The plants were classified and identified botanically and were collected from different parts of Myanmar according to the availability. They were also selected according to the priorities.

Regarding the crude plant extract, some were prepared as watery decoction, according to the reputed informations gathered. Some plants which possessed very succulant leaves were extracted with 95% alcohol so that the extract could be preserved for longer period without contamination of fungus. Some easily air-dried plants were extracted with 50% alcohol. The most active fruit was found to be A. sativa which was edible and was available throughout Myanmar. It was potent and killed the worms within 24 hrs. As shown in Table 2, time taken for 100% lethalities of the worms were expressed in hours. The time taken for most effective two plants and one fungus extracts like A. sativa, U. indica and H. repanda were approximately 17 hrs, 22 hrs and 24 hrs respectively and was found to be significantly ($p<0.005$) less than that of the control group being 118 hrs. So also as for the reference compound, piperazine time taken for 100% death were 13 hrs, 7 hrs and 3 hrs respectively for three dose levels of 4 mM, 8 mM and 16 mM per ml final medium concentration.

The worm became paralysed after about 6 hrs and complete inhibition was observed after 21 hrs. There was no significant stimulatory effect of the drug on the movement. The second active plant U. indica was liable to cause initial stimula-

tion to the parasite resulting in increasing activity or percentage of force of contraction of the worm as well as strengthening the motor power or motility in percentage of frequency of the parasite. Both these unwanted increase in contraction and frequency lasted for 1-3 hr and therefore it has attended risk of causing complication such as intestinal obstruction and perforation to the host when given orally. The third active fungus H. repanda was also effective at a concentration of 16 mg/ml and had a significant actions on the motility and magnitude at 3 hr and onwards when compared to its original control value. In conclusion, it was observed that the 17 plant extracts including 1 fungus extract tested were much less active than the standard drug, piperazine.

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Bronchodilating activity of some traditional medicine formulations on the in vitro tracheal ring test model

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The efficacy of seven reputed bronchodilating traditional medicine formulations were studied on the in vitro model of tracheal chain preparation using rabbit and guinea-pig. A new carbachol-induced model was introduced and comparison of its efficacy with normal preparation showed more promising results. Three formulations were found to produce significant relaxation as much as 127 ± 12 percent and 108 ± 18 percent in rabbit and guinea-pig models respectively.

INTRODUCTION

Traditional medicine formulations (TMFs) TMF-01; TMF-04; TMF-07; TMF-08; TMF-25; TMF-28; TMF-29 and TMF-35A are used in Ayurvedic Indigenous Medicine against respiratory disorders like cough with tightness of chest, dry cough, productive cough, whooping cough, chest pain, purulent bronchitis and asthma [1]. A survey of literature indicates that no systematic investigations on the pharmacology of the TMFs have been carried out. In view of the popular use of these TMFs in asthma, systematic investigations are undertaken to be done experimentally. The most logical and economical approach to varify certain reputed drugs for a particular activity or a set of activities is the biological screening employing suitable in vitro models. In this study tracheal ring test models using guinea-pig and rabbit tracheal rings were utilised for investigation of bronchodilating effects of some famous Myanmar traditional medicine formulations.

MATERIALS AND METHOD

(a) Preparation and extraction

A total number of seven reputed antiasthmatic and expectorative Myanmar traditional medicine formulations (TMFs) used in this study were prepared by the authorized personnels of the Department of Traditional Medicine according to the classical text [1]. Voucher specimen has been deposited at the Pharmacognosy Section, Pharmacology Research Division, Department of Medical Research, Yangon, Myanmar. Ten gramme of TMFs to be tested were allowed to dissolve in 50 ml normal saline for overnight. Then the supernatant was decanted and centrifuged for 15 mins at 2500 r.p.m. (IEC Model CL centrifuge, International Equipment Company, U.S.A.). About 10 ml of clear fluid obtained was evaporated in a watch glass on a water-bath and the weight of substance was determined to obtain the concentration of the soluble portion in the test sample. The effect of water soluble fraction

which was equivalent to 500 mg of the soluble substance was tested.

(b) Preparation of tracheal smooth muscle strips

Tracheal chains of rabbits and guinea-pigs were selected for both test models as each of them represents a different species of animals. Four rabbits (of either sex weighing approximately 1.8 Kg) and four guinea-pigs (of either sex weighing approximately 0.8 Kg) were sacrificed and tracheas were removed from the rabbits and guinea-pigs immediately after sacrifice. The trachealis muscle was dissected free of the adventitial layer and cut into transverse strips [2,3,4]. Tracheal muscle was kept well oxygenated during the dissection.

Segments of trachea each consisting of 3-4 rings of cartilage were dissected. Four to five such segments of tracheal rings were tied with a cotton thread in such a way that the muscular portion were alternately orientated on opposite sides. The strip was mounted in an organ bath of 50 ml capacity filled with Krebs-Henseleit solution kept at 37°C and continuously bubbled with 95% O_2 and 5% CO_2. One end of the tracheal chain was attached to the hook of the gas-supplying tube and the other to a transducer (Force transducer Type 45196, San-Ei Instrument Co., Ltd., Japan) and responses were recorded on a polygraph (141-6; San Ei Instrument, Japan). An appropriate passive tension was applied, and the strips were allowed to equilibrate for approximately one hour. Following the equilibration period, the strips were allowed to contract by adding carbachol (50 mg total) to the organ bath. To determine the effect of the water soluble fraction on the contraction of the trachealis muscle, the volume equivalent to 500 mg of the soluble substance was added to the organ bath consecutively after stimulating the muscle with the contractile agent. The response of the tracheal ring to each dose of the test drugs was recorded for 5 mins. The drug was then washed out and the tissue was allowed to rest for 15 mins or more until the tissue recovered to the base-line. On the same tissue preparation various TMFs water soluble substances were applied. Each formulation was tested at least 10 times and their responses and standard errors were calculated.

RESULTS

Effect on trachealis muscles

Tracing samples of bronchodilating response of TMFs extracts on rabbit trachea preparations were shown in Fig. 1. Dose responses of TMF-01,

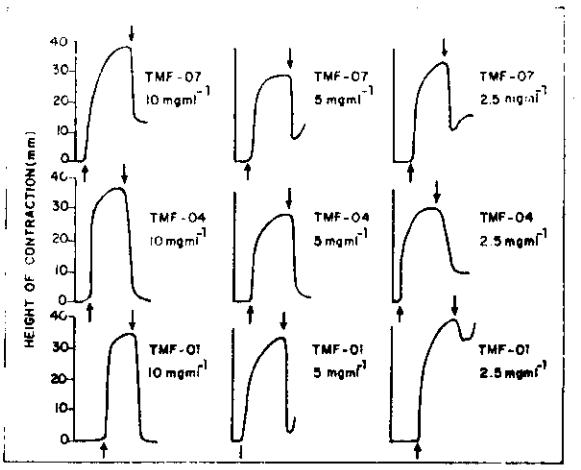

Fig. 1. Tracing samples of bronchodilating response of traditional medicne formulations on tracheal chain preparations.

The magnitude of the illustration is in its original scale as recorded by the polygraph.

The ordinate is the height of contraction in mm.

↑ arrow shows the point of carbachol (50 mg) administration into the muscle bath and ↓ indicates the addition of watery extracts of traditional medicine formulations into the muscle bath.

TMF-04 and TMF-07 were concentration-dependent and reversible. When the TMFs extracts were added to the organ bath after stimulating the rabbit trachealis muscle with carbachol, the three extracts inhibited the contractions and these inhibitions were clearly in a concentration-dependent manner. The magnitude or height of contraction in mm is in its original scale as recorded by the polygraph.

The effect of TMFs extracts on the isolated rabbit and guinea-pig tracheal chain preparations was shown in Table 1. Out of seven TMFs extracts tested, only three TMFs (Table 2,3 and 4) extracts significantly inhibited the contractions induced by the contractile agent. Mean responses of 10 mg ml^{-1} bath concentration of the above three TMFs extracts and aminophylline on both animal species were shown in Fig. 2.

It was found that TMF-01 and TMF-04 10 mg ml^{-1} bath concentration were found to be equipotent to that of 0.625 mg ml^{-1} bath concentration of aminophylline because there were no significant differences within the groups. But it was observed that

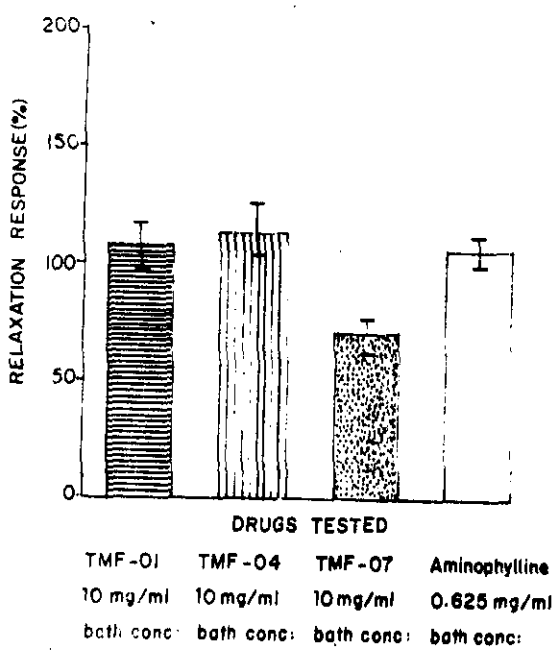

Fig. 2. Effect of traditional formulations on isolated rabbit and guinea-pig tracheal chain preparations.
The abscissa shows the drugs tested. The ordinate shows the mean relaxation response in percent. Vertical bar represents the standard error of the means. Each bar represents the results of (17-24) tests.

the mean response of 10 mg ml^{-1} bath concentration of TMF-07 was slightly lower than that of aminophylline ($p<0.05$).

Table 1. The effect of traditional medicine formulations on the isolated rabbit and guinea-pig tracheal chain preparations

Sr.No.	Traditional medicine formulations tested	Rabbit relaxation response (%)	Guinea-pig relaxation response (%)
1.	Thaleikpoke pyauk hsay; TMF-01	127 ± 12	86 ± 8
2.	Pannar chweyaing hsay; TMF-04	118 ± 3	108 ± 18
3.	Nweigyo-ke chaungzoepyauk hsay; TMF-07	95 ± 9	46 ± 4
4.	Thwaydular hsay; TMF-08	20 ± 7	29 ± 12
5.	Mademyinmogon hsay; TMF-25	18 ± 7	33 ± 9
6.	Thatbazayya hsayzatkyee; TMF-29	2 ± 1	2 ± 1
7.	Zartila winga hsay; TMF-35A	18 ± 7	12 ± 3

Table 2. Ingredients of TMF-01 (Thaleikpoke pyauk hsay)

Scientific/common name	Family	Myanmar name	Part used	Amount in gm%
Major ingredients:				
1. Matural sodium chloride; rock salt	-	Theindaw		31.5
2. Unidentified	-	Metlingyin	Fruit	19.7
3. Ammonium chloride	-	Zawetthar		17.7
4. Potassium aluminium sulphate; alum	-	Kyaukchin		15.7
5. Ammomum subulatum	Zingiberaceae	Pharlargyee	Fruit	7.9
6. Elettaria cardamomum	Zingiberaceae	Pharlarngei	Seed	3.9
7. *Zingiber officinale*	*Zingiberaceae*	Gyingyauk	*Rhizome*	*2.0*
8. Piper longum	Piperaceae	Peikchinn	Fruit	0.5
9. Piper nigrum	Piperaceae	Ngayokkoung	Fruit	0.5
Other plant ingredients:				
10. Anethum sowa	-	Samonnyo	Fruit	0.1
11. Foeniculum vulgare	*Umbelliferae*	*Samonphwe*	Stem	0.1
12. Foeniculum vulgare	Umbelliferae	Samonsabah	Fruit	0.1
13. Lepidium sativum	Cruciferae	Samonni	Seed	0.1
14. Trachyspermum ammi	Umbelliferae	Samonbyu	Fruit	0.1

San-Khin [8], Hundley and Chit-Ko-Ko [9]

Table 3. Ingredients of TMF-04 (Pannar chweyaing hsay)

Scientific/common name	Family	Myanmar name	Part used	Amount in gm%
Major ingredients:				
1. Sodium chloride	-	Hsah yoeyoe		59.3
2. Citrus medica	Rutaceae	Thanbayar	Fruit juice	37.1
Other plant ingredient:				
3. Piper betle	Piperaceae	Kun	Leaf juice	3.0
Other mineral/salt ingredient:				
4. Arsenic sulphides; red arsenic	-	Seinni myinthwah		0.6

San-Khin [8], Hundley and Chit-Ko-Ko [9]

Table 4. Ingredients of TMF-07 (Nweigyo-ke choungzoepyauk hsay)

Scientific/common name	Family	Myanmar name	Part used	Amount in gm%
Major plant ingredient:				
1. Glycyrrhiza glabra	Papilionaceae	Nweigyo	Stem	23.0
Other mineral/salt ingredients:				
2. Ammonium chloride	-	Zawetthar		23.0
3. Borax	-	Letchah		23.0
4. Natural sodium chloride; rock salt	-	Theindaw		23.0
Other plant ingredients:				
5. Anethum sowa	Umbelliferae	Samonnyo	Fruit	1.1
6. Foeniculum vulgare	Umbelliferae	Samonsabah	Fruit	1.1
7. Foeniculum vulgare	Umbelliferae	Samonphwe	Peduncle & stem	1.1
8. Kaempferia galanga	Zingiberaceae	Kunzah gamon	Rhizome	1.1
9. Lepidium sativum	Cruciferae	Samonni	Seed	1.1
10. Santalum album	Santalaceae	Nathabyu	Wood	1.1
11. Trachyspermum ammi	Umbelliferae	Samonbyu	Fruit	1.1

San-Khin [8], Hundley and Chit-Ko-Ko [9]

DISCUSSION

There are two possible methods of tracheal chain preparation to detect relaxant activity of various drugs namely Castillo and De Beer [2] method and Niewoehner et al. method [3] and Aqel and Hadidi [4]. The former one is the oldest method and simple to prepare which allows relaxation to be obtained without prior addition of any spasmogen, whereas in the latter are the tissue has to be induced with a spasmogen prior to the test, and seems to be more complicated. Nevertheless the latter one more resembles the pathology found in clinical asthmatic attack. In this study the carbachol-induced method is found to be more effective and applicable. Implication is that this method for screening the reputed Myanmar antiasthmatics and expectorants provides adequate sensitivity to measure the relaxant effects.

By using the carbachol-induced method three traditional medicine formulations were demonstrated to be good bronchodilators among seven tested TMFs. All three TMFs (TMF-01, TMF-04 and TMF-07) were found to relax the tracheal preparation in a manner similar to aminophylline. Relaxation of tracheal muscle may be due either to direct action or through adrenergic receptors. The adrenergic receptors of trachea has been described to be predominantly of the beta type as stated by Ahlquist [5] and Foster [6]. However, the mechanism of action has not been studied in the present study. The large standard errors of the means may be due to the large variation in response within preparations. These preparations are very delicate and fragile

and are very sensitive to fluctuation in temperature, change of oxygen and carbon dioxide content in muscle bath solution and improper handling of tissue during dissection, or inadequate resting period for tissue to regain full inherent tone.

According to the Ayurvedic texts the immature fruits and roots of Piper nigrum, a constituent of TMF-01, were used in chronic bronchitis, cough and cold; Piper betle, a constituent of TMF-04, was used in respiratory catarrh. In addition, it was also reported to dispel flatus, stop coughing, reduce inflammation and alleviate itching. Lepidium sativum was used in asthma and cough with expectoration. However, the active principle of any of these agents has never been isolated nor tested to determine the mechanism of action. The present study appears to be an appropriate way to evaluate the bronchodilating activity of drug mixtures whose active principles are unknown and yet to be isolated. It also serves as a preliminary screening test for this purpose.

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Detection of hepatitis E virus genome with a thymine-thymine dimerized cDNA probe

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The study described the spot hybridization technique using a non-radiolabeled, thymine-thymine dimerized synthetic oligonucleotide complementary DNA(cDNA) probe for the detection of hepatitis E virus genome in bile. The test performed well with high degree of sensitivity and specificity. It was compared with the conventional electron microscopy and was found to be thousand times more sensitive.

INTRODUCTION

Spot hybridization using synthetic oligonucleotide probes is a uniquely powerful and sensitive assay in which a minute amount of genomic material in a sample can be detected. Recently the technology has been successfully applied to various samples for detection of a large number of viruses including hepatitis B virus [1] and human immunodeficiency viruses [2]. The thymine-thymine (T-T) dimerized cDNA probes are advantageous in terms of probe stability, personal safety and simple technique [3]. This report demonstrates the successful application of non-isotopic T-T dimerized cDNA probe in spot hybridization for the detection of hepatitis E virus (HEV) genome in the bile of monkeys which have been serially passaged with HEV.

MATERIALS AND METHODS

Animal transmission and sample collection

Animal transmission of HEV in monkeys have been described [4]. Bile was collected through a minilaparotomy weekly after inoculation for 4 weeks. In this experiment bile collected from the 5th passage monkey on post first, second and third weeks were used. Preinoculation bile was also collected for sample control.

Electron microscopic examination of bile was described previously[5].

cDNA probes

An antisense probe which is a complementary DNA (cDNA) to a thirteen base pairs sequence of a portion of HEV genome [6] was

synthesized to use as a test probe. A sense probe which has complementary base pairs sequence to the antisense probe, in other words, it has identical base pairs sequence to the HEV genome was also synthesized and used as a control probe. Both sense and antisense probes were irradiated with uv light to produce T-T dimerized probes [3].

Spot hybridization

50 ul of bile was diluted 1:40 with phosphate buffered saline (PBS), mixed and ultracentrifuged (Hitachi Koki Co. Ltd. rotor RP 50-2-423) at 40,000 rpm at 4°C for 2 hr. The virus pellet was dissolved in 50 ul of Tris-EDTA solution and spotted on the nitrocellulose paper (4 ul/spot). The membranes were dried at room temperature and digested with proteinase K solution (1ugm/ml in PBS) for 10 min at 37°C. They were then washed three times with PBS to remove proteinase K. After washing the membranes were prehybridized at 42°C overnight. Hybridization was performed seperately with T-T dimerized sense and antisense probes at 42°C for 2 hr. After hybridization they were reacted 3 hr with rabbit serum against T-T dimers of the probes at the predetermined optimum dilution at room temperature. Washed several times in PBS and reacted again with commercial horseradish peroxidase labeled goat anti-rabbit immunoglobulins (MBL, Japan). Color production was performed in the presence of diaminobenzidine, hydrogen peroxide, cobalt and nickel [3].

RESULTS

As shown in Fig. (1) positive stainings were seen in all 3 postinoculation bile collected at first, second and third weeks. The reaction was specific since it did not react with preinoculation bile.

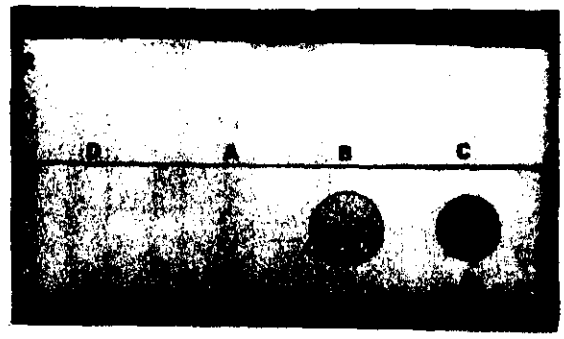

Fig. 1. Spot hybridization results of bile and synthetic hepatitis E virus probes. Post 1,2,3 weeks after inoculation bile were spotted at A,B & C positions of both papers respectively. At Ds bile from uninoculated monkey was spotted. Paper 1 & 2 were hybridized with test and control probes respectively. Positive staining were seen in A,B & C of paper [1].

Also there was no hybridization between the control (sense) probe and postinoculation bile samples.

The technique is more sensitive than the conventional electron microscopy, as the bile at post one week which is negative for virus particles under electron microscope gave positive reaction. The sensitivity of the technique was further, evaluated by using 1:10, 1:100, 1:1000, 1:10000 dilutions of bile which contained virus particles +1 under the electron microscope. Virus positive bile was semiquantitatively graded from +1 to +3. +1 means only a single virus particle was detected in all 400 squares of the grid. +2 denotes more than one virus usually in groups of 2 or 3 per 100 squares. +3 is more than +2.

Fig. (2) is the spot hybridization results of the serially diluted bile of post one week. Positive staining was obtained up to 1:1000 dilution. Thus the technique is at least 1000

Fig. 2. Electron micrograph of bile showing a single virus-like particle (arrow).

times more sensitive than the conventional electron microscopy.

DISCUSSION

The data presented have shown that T-T dimerized HEV cDNA probes performed well and it confirmed that the virus like particles visualized under the electron microscopy were the causative virus of HEV. We have demonstrated the specificity of the technique by including sample control as well as probe control.

The sensitivity of the assay was compared with that of conventional electron microscopy. It was found to be superior and in fact 1,000 times more sensitive than the conventional electron microscopy. This is in agreement with others working on hepatitis A virus. They have reported that molecular hybridization was more sensitive than the immune electron microscopy [7]. It is also known that immune electron microscopy is more sensitive than the conventional electron microscopy [8].

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The effect of halofantrine versus quinine on patients with uncomplicated falciparum malaria in Myanmar

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Sixty patients with uncomplicated falciparum malaria (less than 5% of RBCs parasitised) were studied. Patients were alternatively assigned to treat with Halofantrine or Quinine. Halofantrine was given orally 500 mg six hourly for three doses. Quinine 10 mg/kg was given orally three times a day for 7 days. All the patients were admitted to the hospital for 7 days and were followed up on days 14, 21 and 28. There were no differences in parasite clearance time and fever clearance time between two groups of patients. Among the patients treated with Quinine two failed to complete the drug course. In one patient who was treated with Halofantrine, the initial parasite count was 171429 cumm at 0 hour of admission to hospital. The parasites were cleared at 42 hours and he became unconscious at 48 hours. He was put on standard dose of Artemether plus Mefloquine, but the patient became deeply jaundice and expired at 72 hours of admission to hospital. We concluded that further studies are needed to evaluate the efficacy of Halofantrine on Myanmar patients with complicated falciparum malaria.

INTRODUCTION

Halofantrine belongs to a new class of compound, the phenanthrene methanols. It provides fast and effective treatment for all forms of malaria particularly that caused by both sensitive and multi-drug resistant strains of P. falciparum. Preliminary clinical study suggested adult dose of 500 mg 6 hourly for 3 doses is effective. In an on going clinical study, only 0.6% of the patients with P. falciparum failed to clear the parasitaemia within 7 days. Recrudescence of parasitaemia occured in 6% of patients [1]. But recent study from Thailand had reported much lower cure rate, hence this study is needed.

PATIENTS AND METHODS

The study was conducted during 1991 malaria season at Tharyarwady Civil Hospital which is situated in the west of Bago Yoma, 128 km north of the Yangon, where resistance of P. falciparum to Chloroquine, Sulfadoxine Pyrimethamine had been reported. Sixty patients with uncomplicated falciparum malaria were studied.

Inclusion criteria

Patients with uncomplicated falciparum malaria who were free from anti-malarials in urine at the time of admission and given consent to take part in the trial were included in the study. Only patients age

above 12 years were included in the study.

Exclusion criteria

Patients with one or more of the following complications were excluded from the study.

(a) More than 5% of RBCs parasitised
(b) High fever over 105°F
(c) Severe anaemia (PCV less than 20%)
(d) Blood urea above 50 mg/dl
(e) The presence of clinically detectable jaundice
(f) Those with cerebral signs and symptoms
(g) Patients under 12 years of age and pregnant women.

Method of treatment

The patients were divided into two groups and were alternately assigned to one of the following treatment regimens.

Regimen (1): Study group

Halofantrine hydrochloride tablets manufactured from Smith Kline and French was given orally 500 mg six hourly for a total of three doses.

Regimen (2): Control group

Quinine tablet (manufactured from Myanmar Pharmaceutical Factory) 10 mg/kg body weight was given every 8 hourly for 7 days.

All patients were admitted to the hospital for a minimum of 7 days. Body temperature was recorded every six hourly until normal and every 12 hourly thereafter. Thin and thick blood films were taken every six hourly until no asexual form have been seen in blood for 24 hours, then daily upto day 7 and subsequently on days 14, 21 and 28. Routine haematology and biochemical examinations were also done at days 0, 3 and 7.

RESULTS

Among patients treated with Halofantrine one patient expired. The expired patient's initial parasite count was 171429 cumm at 0 hour of admission to hospital. Though the parasites were cleared at 42 hours. the patient became unconscious at 48 hours. He was put on standard dose of Artemether and Mefloquine. He became deeply jaundiced and expired at 72 hours of admission to hospital.

There were no serious clinical and biochemical changes at the remaining patients.

In the Quinine group, signs and symptoms of drug intolerance such as nausea, vomitting, giddiness and tinitus occurred in (16) out of (30) patients. Two patients refused to continue to take quinine course because of above symptoms.

There were no significant differences between the parasite clearance time and fever clearance time of two groups of patients.

There was no recrudescence of parasitaemia among the 20 patients treated with Halofantrine and followed till day 28. But among the 23 patients treated with Quinine. One patient recrudesce at day 21 recrudescence rate was 4.3% (Table 1 and Figure 1).

DISCUSSION

In this study, the patients treated with halofantrine were free from signs and symptoms of drug toxicity. However, the International study [1] as well as the two other studies from Myanmar [2,3] had reported no remarkable clinical side effects and

Table 1. Effect of Halofantrine versus quinine on patients with uncomplicated falciparum malaria

Groups of patients studied	No. of patients studied	Average parasite clearance time (Mean ± SD)	Average fever clearance time (Mean ± SD)	Mortality rate	Recrudescence rate (Till day 28)
Halofantrine	30	57.69 ± 6.88	41.08 ± 11.25	3.3%	0% *
Quinine	28	57.00 ±24.80	44.80 ± 32.70	0%	4.3% **

N.B: * Recrudescence rate calculated on 20 patients

** Recrudescence rate calculated on 23 patients

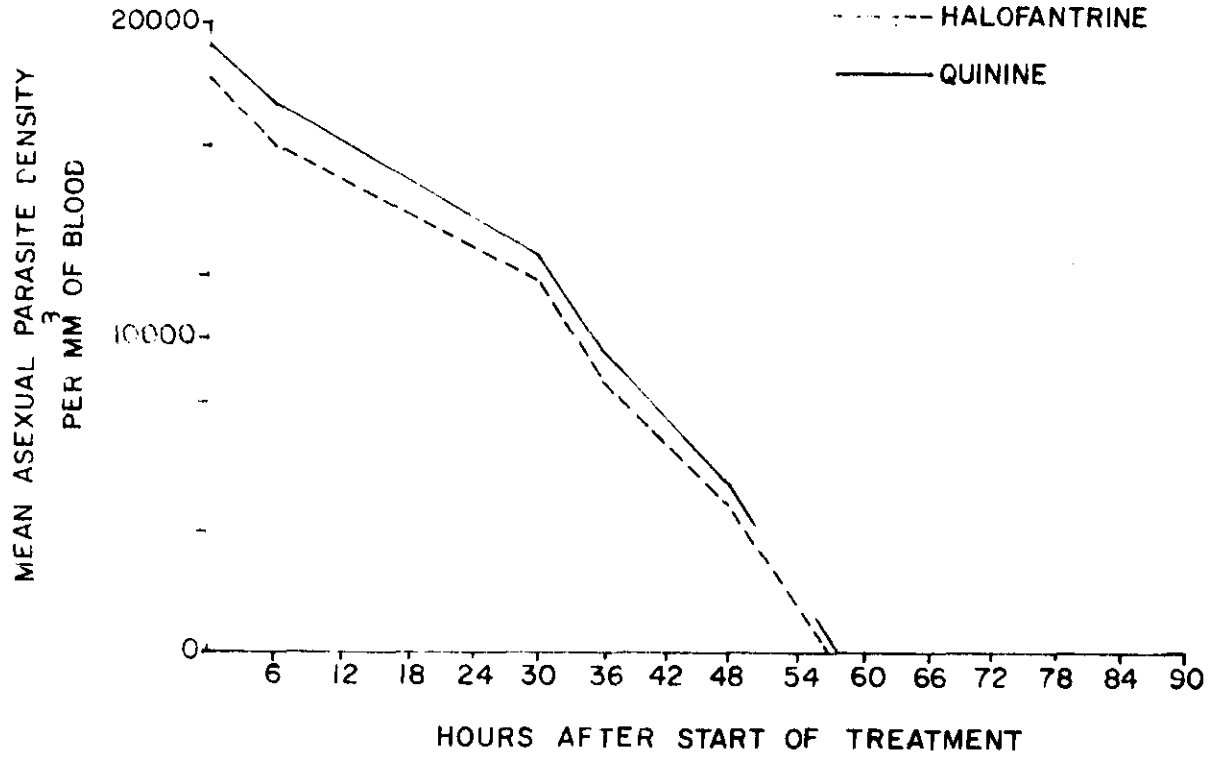

Figure 1. The effect of drugs on the rate of the parasite clearance

laboratory changes associated with the Halofantrine. Hence drug toxicity may not be a problem associated with Halofantrine. But among the patients treated with Quinine 53.3% were associated with cinchonism and two patients failed to complete the course.

In this study, there was no recrudescence of parasitaemia among patients treated with Halofantrine, Kyaw Win et al. [2] reported recrudescence in only one patient at day 36 among 22 patients studied. Ye Thwe et al. [3] had found no recrudescence on 23 patients who were followed till day 28. Horton et al.[1] had reported the recrudescence rate of 6% out of the large sample of the patients studied. Hence, recrudescence may not be a problem among the patients treated with Halofantrine.

In this study, the mean parasite clearance time and fever clearance time of the patients treated with Halofantrine were comparable to Quinine. Our data was also comparable to the results of other study where Horton et al. [1] had reported the fever clearance time (FCT) and parasite clearance time (PCT) as 57.9 hours and 50.2 hours respectively.

Initial study on 22 Myanmar adult patients with uncomplicated falciparum malaria and treated with above dosage of Halofantrine had cured all the patients [2]. In the International trial [1] only 8 (0.6%) out of 1315 evaluable patients with P. falciparum failed to clear the prasitaemia in 7 days. In our study, one patient expired. Though the parasites were cleared from circulation at 42 hours, the patient became comatose at 48 hours and of admission to hospital and died at 72 hours. Ye Thwe [3] had also reported the shot up of the parasitaemia in one patient from 42,000/cmm to221,000/cmm after 3 doses of Halofantrine at 20 hours of treatment. Initial field trial with halofantrine on the Thai-Cambodian border in 1982 showed 88% cure rate with the above dosage [4]. In a more recent trial, the cure rate was only 28% for halofantrine [5]. This low cure rate may be due to the low and variable absorption of halofantrine hydrochloride [6]. It may be also be due to the development of partial resistance of P. falciparum to this drug as suggested by Schildbach [7]. In vitro studies carried out in Myanmar had also indicated that 10-17% of isolate were probably resistant to the drug [3,8]. Thus the dosage of halofantrine may be needed to be reconsidered and parenteral compounds are also needed for use on patients with severe and complicated malaria. We conclude that further studies are needed to evaluate the place of halofantrine on Myanmar patients with severe and complicated falciparum malaria.

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Identification of Escherichia coli toxins by Bead ELISA

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Seven hundred and eleven isolates of Escherichia coli and 21 isolates of other bacteria were tested for the production of toxins as shiga-like toxin 1 (SLT I or VT_1), shiga-like toxin 2 (SLT II or VT_2) and heat labile toxin (LT) by Bead ELISA method. It was observed that 31 percent of children and 50 percent of adults with diarrhoea and gastroenteritis were caused by enterotoxin. The verotoxin E. coli strains belonged to serogroups 026, 055, 086, 0125, 0142 and 0157 and other untypable serogroups.

INTRODUCTION

It has long been known that certain strains of Escherichia coli can cause acute diarrhoea in man and animals. The organism has been implicated in cases of clinical cholera. Enteropathogenic strains of E. coli when injected into ligated ileal loops, behave like V. cholerae in causing fluid accumulation, and this enterotoxin effect can be reproduced with cell free preparation from culture fluids. Further work has revealed that most enteropathogenic strains of E. coli produce that labile enterotoxin (LT) and sometime also elaborate distinguishable heat-stable enterotoxic factor (ST). Production of both toxins is mediated by transmissible plasmids and thus organism carrying these plasmids may be able to transfer enterotoxigenicity to other Gram-negative bacterial species. Moreover, there are certain strains which produce enterotoxin similar to that of Shigella dysenteriae type 1 and indicated that some of these were LT negative, ST negative and neutralized well with anti Shiga toxin and known as VT1 of SLT1. However, the toxins that were not neutralized by the anti-Shiga toxoid could be divided into two groups on the basis of their reaction with the convulescent serum, some that were partially neutralized (presumably contained VT2 or SLT II) and others that were not neutralized at all. Some workers also reported that some E. coli strains produce verotoxin but not identical to the prototype SLT II. Such strains contain an extra-cellular verotoxin that, unlike LST II, is not active on Hela cells, but is neutralised by reference antisera against SLT II was reported by Marques et al., 1987 [1], Karmali et al., 1986 [2] and Smith et al., 1983 [3] and still remain to be determined.

MATERIALS AND METHODS

Organisms tested

A total of 152 organisms isolated from Yangon Children's Hospital and 580 organisms isolated from 108 adult cases with gastroenterititis (including 9 dysentery cases) were tested for the production of toxins by Bead ELISA method. From the cases of children 5 isolates of Plesiomonas, 10 strains of Salmo-

nella, 3 strains of Vibrios, 2 strains of Edwardsiella and one strain of Shigella were also included.

Assay of cytotoxicity by vero cells

Cytotoxicity to vero cells was assayed as described by Konowalchuk, 1977 [4] and Scotland et al., 1980 [5]. Vero cells were grown in Eagle's Minimum Essential Medium with glutamine supplemented with 2 percent foetal calf serum. Toxin activity was assayed in 96 well microtitre plates Falcon 3070 or 3072. Three days prior to assay, vero cells were cultured in the media to 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. It was obtained by shaking vigorously at 280 rpm/min in a shaking incubator overnight at 37°C. For each supernatant duplicate wells were used. The plates were incubated at 37°C with 5 percent carbon dioxide. Positive and negative controls were also included as described in Mar Mar Ny in et al. [6].

Preparation of bacterial toxins and determination by Bead ELISA

E. coli were cultured in 2 ml L broth containing Bactotryptone (1 percent), Bacto yeast extract (0.5 percent) and sodium chloride (1 percent), pH 7.00 in deionized distilled water. It was then shaken in a shaking incubator at 37°C with 280 rpm overnight. Then the cells were sonicated by Tomy Seiko ultrasonic disrupter (Model UK 200P) on ice and spinned at 10,000 rpm for 10 minutes. The 0.25 ml of supernatant was mixed with polysterene beads coated with anti LT, anti VT1 and anti VT2 in the respective tube. Then 0.25 ml of (Buffer A) which constitutes 10 mM sodium phosphate with 0.1 M sodium chloride, 0.1 percent sodium axide and 0.1 percent Bovine serum albumin (pH 7.0) was added into the tubes for incubation at 37°C for 1 hour. The polysterene beads were washed twice with 0.01 M sodium phosphate buffer (pH 7.0) containing sodium chloride (Buffer B). The bead was then incubated at 37°C for 1 hour with Fab'-horse radish peroxidase conjugate (200 mg/ml) in buffer B containing 2 percent bovine serum albumin. After incubation, the polystene bead was washed twice with buffer and the beads were transferred to new test tubes.

Peroxidase activity bound to polysterenes bead was then assayed by adding 0.56 mM 3,3',5,5' tetramethyl benzidine (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 percent hydrogen peroxide was added and incubated at 30°C for additional one hour. The reaction was stopped by adding 0.2 ml of 4 N sulphuric acid and the resulting yellow color was measured at 450 nm in a spectrophotometer as described by Oku et al. [7].

RESULTS

E. coli isolates that produce either VT1 or SLT1; VT2 or SLT II or LT were shown in Table 1. It was found that a total of 103 isolates of E. coli (36 isolates from children and 67 isolates from adults) were detected to produce toxin(s). 18 isolates produce VT1 or SLT I toxin; 29 isolates produce VT2 or SLT II toxin; 35 isolates produce heat labile toxin (LT); 7 isolates produce VT1 and VT2 toxins, 6 isolates produce VT I and LT toxins; 5 isolates produce VT2 and LT; and 3 isolates produce VT1, VT2 and LT toxins. It was observed that 31

Table 1. VTEC strains isolated from children and adult cases

Toxin	*Children*		*Adults*		*Total*	
	Case study 100	Organism isolated 152	Case study 108	Organism isolated 580	Case study 208	Organism isolated 732
VT1	8	10	8	8	16	18
VT2	2	2	18	27	20	29
LT	16	19	14	16	30	35
VT1 VT2	2	2	3	5	5	7
VT1 LT	1	1	5	5	6	6
VT2 LT	2	2	3	3	5	5
VT1 VT2 LT	0	0	3	3	3	3
Total	31 (31.0)	36 (23.7)	54 (50.0)	67 (11.6)	85 (40.9)	103 (14.1)

Figures in parenthesis denote percentages

percent from children and 50 percent from the adult of E. coli cases produce toxin(s).

Table 2 represents the relationship between serogroups and types of verotoxic E. coli strains. In cases of children, 1 isolate of serogroup 08 showed VT1 or SLT I; 1 isolate of 025 showed VT2 or SLT II; one isolate of 026 showed VT1; 2 isolates of 0125 showed VT1 or SLT I, one isolate of 0159 showed VT2 or SLT II. From 11 isolates of untypable E. coli 7, 2 and 2 cases showed VT1; and VT1 VT2 respectively, one strain of Plesiomonas also showed production of VT1 (SLT I). From the adult cases, 1 isolate of serogroup 055 showed VT2; one isolate of serogroup 086 showed VT2, one isolate of 0142 showed VT1; 2 isolates of 0157 which was from dysentery case showed VT1 and VT2 each. In 46 isolates of untypable E. coli 11 isolates showed VT1, 27 isolates showed VT2 and 8 isolates showed VT1 and VT2.

Table 3 shows the VTEC isolated from children with diarrhoea and control cases. It was found that in 31 cases (20 from diarrhoea and 11 from control) showed to produce toxin(s).

Table 2. Serotypes and types of VTEC isolates

Serotype of VTEC	Verocytotoxin type	No. of isolates	Produce Bloody diarr.	Produce Diarr
08(ST)	VT1	1		1
025(ST)	VT2	1		1
026	VT1	1		1
0125	VT1	2		2
0159(ST)	VT2	1		1
UT	VT1	7		7
	VT2	2		2
	VT1 VT2	2		2
Plesiomonas	VT1	1		1
Total		18		18
055	VT2	1		1
086	VT2	1		1
0142	VT1	1		1
0157	VT1	1	1	
	VT2	1	1	
UT	VT1	11		11
	VT2	27	5	22
	VT1 VT2	8	1	7
Total		51	8	43

It was also observed that there is no significant difference between the diarrhoea and control group. However, it was also found out that among the 11 control cases, 9 cases

Table 3. VTEC isolated from children with diarrhoea and control cases

Type of Toxin	1 - 3 yr			
	Diarrhoea		Control	
	Cases tested 61	Org. isolated 96	Cases tested 39	Org. isolated 56
VT1	3	3	5	7
VT2	2	2	0	0
LT	12	15	4	4
VT1 VT2	1	1	1	1
VT1 LT	1	1	0	0
VT2 LT	1	1	1	1
VT1 VT2 LT	0	0	0	0
Total	20 (36)	23 (24)	11 (28)	13 (23)

Figures in parenthesis denote percentages

were associated with one or more pathogens such as ETEC, Plesiomonas, Vibrio or salmonella. Thus only 2 cases left without any association with other pathogens.

Table 4 shows the isolation rate of VTEC from adults with diarrhoea and bloody diarrhoea. It was found that out of 9 dysentery cases 37 organism were tested; one case was found out to be Shigella and the other 4 cases produced one VT1, 2 VT2 and one VT1VT2. In the diarrhoea cases, Shigella was isolated from 5 cases, 7, 17 and 2 cases showed VT1, VT2 and VT1VT2 respectively. In total 5 out of 9 cases (55.6 percent) from dysentery cases and 41 out of 99 cases (41.4 percent) of diarrhoea cases were caused by VTEC or Shigella respectively. Table 5 shows the correlation between Bead ELISA and cytotoxicity by Vero cell assay method. Out of 18 strains of E. coli tested 16 strains showed vero cell cytotoxicity i.e., 88.9 percent. Besides, there were also vero cell positive but ELISA

Table 4. VTEC isolated from adults with diarrhoea

Type of Toxin	Bloody diarrhoea		Diarrhoea	
	Cases tested 9	Org. isolated 37	Cases tested 99	Org. isolated 543
VT1	1	1	7	7
VT2	1	1	17	26
LT	1	1	13	15
VT1 VT2	1	1	2	4
VT1 LT	0	0	5	5
VT2 LT	1	1	2	2
VT1 VT2 LT	0	0	3	3
Total	5 (56)	5 (14)	49 (49)	62 (12)
Shigella	1	5	5	16

Figures in parenthesis denote percentages

Table 5. Correlation between Bead ELISA and cytotoxicity by cell culture method

Type of toxin	Bead ELISA	Vero cell
VT1	12	10
VT2	4	4
VT1 VT2	2	22
Total	18	16(88.9%)

negative. Thus needs further clarification.

DISCUSSION

Verocytotoxin was first described by Konowalchuk et al., 1977 [6] who showed that certain strain of E. coli produced a sytotoxin active on vero cells. Two types of verocytotoxin have been established VT1 or SLT I and VT2 or SLT II by Obrien and Holmes, 1987 [8] and Scotland et al., 1985 [9]. VT1 was neutralized by an antiserum to Shiga toxin, produced by strains of S. dysen-

teriae type 1 was demonstrated by O'Brien et al., 1982 [10], whereas VT2 was not neutralized by antishiga toxin or anti VT1. Kleanthous et al., 1985 [11] had shown that the VTEC strains belonged to serogroups 026, 0111, 0113, 0121, 0145 and 0157.

Karmali et al., 1985 [12] also described a method for detecting low numbers of verotoxin producing E. coli in mixed culture by a combination colony 'sweaps' and polymyxin extraction with cell pellets and reported the evidence of VTEC infection in 75 percent of cases. A colony enzyme-linked immunosorbent assay that permits direct detection of high level SLT I producing E. coli on agar plates was described by Strockbine 1986 [13].

Ito et al., 1990 [14] reported the cloning and nucleotide sequencing of verotoxin 2 variant genes from E. coli 091:H21. The termed VT2 variant (VT2v) was found to be similar to VT2 which showed partial immunological identity with VT2 on Ouchterlony test; it has a significantly different isoelectric point from VT2; and it has much less effect on Hela cells than on verocells was reported by Oku, 1989 [15]. Milford et al., 1990 [16] reported that 62 percent of stools obtained early in the course of disease were positive for verocytotoxin or VTEC confirm the importance of the organism in the aetiology of the disease in Haemolytic Uraemic Syndrome (HUS). As the excretion of VTEC is brief as reported by Wells 1983 [17] and diarrhoea has often waned by this time patients are admitted with HUS and these study may be understimate the true association with VTEC. It is therefore difficult to be certain which patients, if any, were truly VTEC negative, and to ascribe specific clinical features to VTEC infection. They found a greater number of deaths in patients positive for VTEC whose stool were analysed within the first week of diarrhoea. However, there are no other differences in the clinical features of patients positive or negative for VTEC.

With the recent emergency of S. dysenteriae type 1 as the predominant pathogen in some tropical regions, the incidence of HUS has increased reported by Raghupathy et al., 1978 [18]. S. dysenteriae type 1 elaborates a toxin that is functionally and structurally identical to VT1 was reported by O'Brien, 1983 [19] and similar to VT2. Kleanthous et al., 1990 [11] reported that they had found 94 percent of VTEC were VT2 producing organisms which is similar to the findings of Scotland et al., 1988 [20] Bitzan et al., 1988 [21] and Tarr et al., 1989 [22], but the implication of this are unclear. Parallels therefore appear to exist between haemolytic uraemic syndrome and VTEC associated diseases in temperate zones and was reported by Raghupathy et al., 1978 [18], O'Brien et al., 1983 [19] and Rahaman et al., 1975 [23]. Their studies confirm and illustrate the importance of VTEC producing organisms. Kleanthous et al., 1990 [11] also reported that 31 percent of children with HUS also produced VTEC, 38 VTEC belonged to serotype 0157H7 or 0157H-, 34 produced VT2 only and 4 strains produced both VT1 and VT2. The remaining 15 VTEC belonged to 9 different 0 serogroups.

VTEC can cause serious human disease but little is known about the sources of the VTEC strains. In North American foods of animal origin appear to be a major source of 0157:H7 infecting humans and outbreaks of haemorrhagic colitis

have been associated with the consumption of hamburger and unpasteurized milk as reported by Riley, 1977 [24] and Karmali, 1989 [25]. Evidence for bovines being a reservoir for 0157:H7 also come from the isolation of these bacteria from cattle in Canada, USA, Argentina and Britain was reported by Karmali, 1989 [25] and Chapman et al., 1989 [26]. Examinations of retail fresh meats and poultry for 0157:H7 in the USA and Canada showed that these organisms were present in 1.5 to 3.5 percent of samples of beef, pork poultry and lamb and it was reported by Doyle et al., 1987 [27]. Thus there a need to establish whether VTEC infections in man are zoonotic in origin and to trace the vehicles of transmission.

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Geographical variation of the biological properties of Russell's viper venom: A Preliminary report

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Venoms from 4 localities (Daik-U, Kungyankone, Htantabin and Htaukkyant) were studied by SDS-PAGE electrophoresis. Biological properties of venom such as lethality, haemorrhagic, necrotic, coagulant, defibrinogenating, capillary permeability increasing and phospholipase activities were carried out according to WHO recommended techniques and standard methods. Venom from Daik-U locality was most lethal, haemorrhagic and necrotic compared to others. It possessed potent defibrinogenating, capillary permeability increasing and phospholipase (indirect haemolysis) activities as well. Kungyankone venom possessed potent coagulant and phospholipase activities with poor defibrinogenating and lethality. Htaukkyant and Htantabin venoms have similar biological properties except the former has poor phospholipase activity. SDS-PAGE electrophoresis and transblotting experiment of venoms showed quantitative difference in numbers of protein band among them. Geographical variation of venom may be responsible for the discrepancy in clinical features observed following Russell's viper bites in different localities.

INTRODUCTION

Monospecific antivenom against Russell's viper prepared by Myanmar Pharmaceutical Industry was used for treating Russell's viper bite patients throughout Myanmar. There were often reports that inspite of antivenom therapy, clot restoration time took longer than 6h and haemorrhagic manifestation persisted for days (Unpublished observation). Geographical variation in biological properties of venom in other species of snake including V. russelli of India has been observed [1-5]. These observation prompted us to study geographical variation of the biological properties of Russell's viper venoms from four available localities.

MATERIALS AND METHODS

Biological properties of venom

The length of the snakes from 4 localities (Figure 1) were measured, milked individually, lyophilised and pooled according to the locality. Biological properties of venoms such as classical LD_{50}, haemorrhagic (MHD), necrotic (MND), and defibrinogenating dose (MDD) were determined according to WHO recommended techniques [1]. Minimum coagulant dose (MCD) was determined by performing plasma recalcification time. Capillary permeability increasing (MCPID) [6] and phospholipase activity (indirect haemolysis) (MIHD) [7] were also carried out.

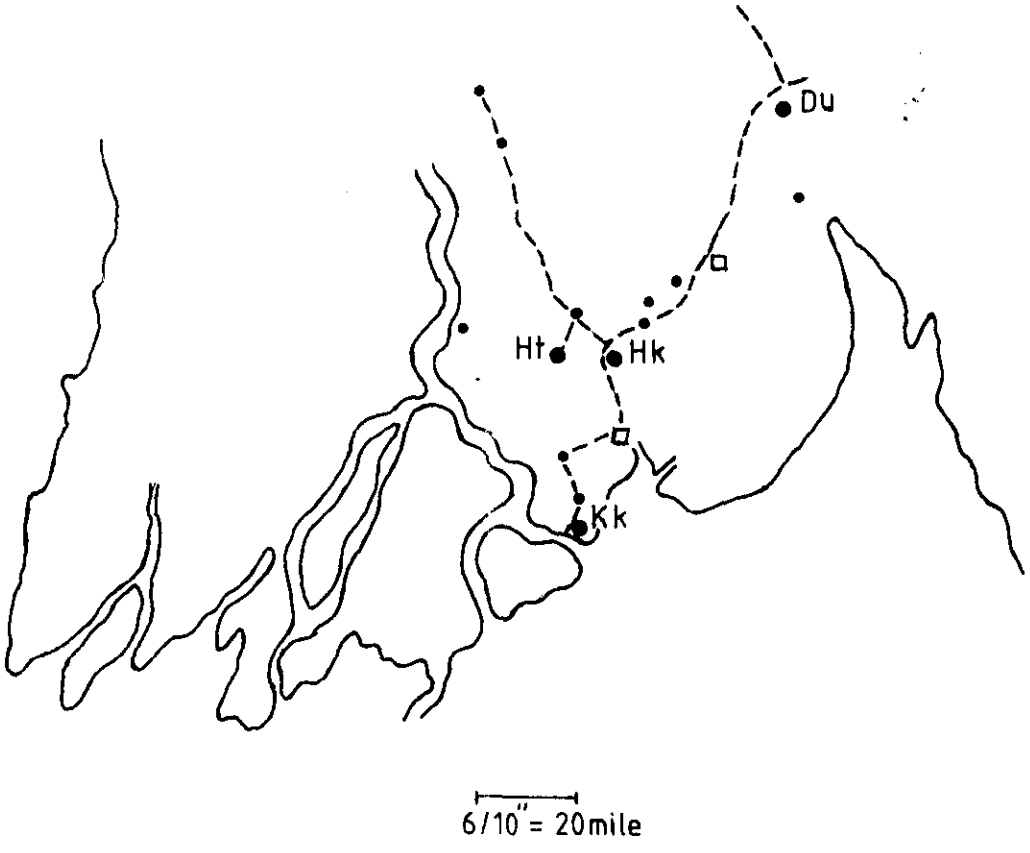

Fig. 1. A map of 4 localities where venoms were available for study.
Du = Daik-U, KK = Kungyankone, HT = Htantabin and HK = Htaukkyant

Plasma recalcification time (MCD)

Briefly, 0.1 ml of venom solution was added to a glass tubes (12 x 75 mm) containing 0.1 ml of normal pooled human citrated plasma and incubated for 2 min at 37°C water bath, after which 0.1 ml of 25mM calcium chloride was added, the tube mixed and the clotting time recorded. Plasma recalicification time (MCD) was calculated by plotting clotting time against venom concentration and reading off the level at the 60 second clotting time.

Capillary permeability increasing dose (MCPID)

The MCPID is defined as the least amount of venom which, when injected intradermally into rats, results a venom lesion of 10 mm diameter 20 min later.

0.1 ml of different concentrations of venom were injected intradermally into the shaved dorsal skin of 200-250 g wister rats under light anaesthesia. Then, 1.5 ml of 1% Evan blue solution was injected into the tail vein of rat. After 20 min,

animals were sacrificed, the skin removed and the diameter of the lesion measured on the inner surface of the skin. The mean diameter of the venom lesion was calculated for each venom dose and MCPID was estimated by plotting mean lesion diameter against venom concentration and reading off the dose corresponding to a 10 mm diameter.

Phospholipase A2 activity (minimum indirect haemolytic dose) (MIHD)

Phospholipase A2 activity was estimated as follow. To 17 ml of 0.6% agarose solution, 0.17 ml of 0.01M calcium chloride, 0.2 ml of egg yolk suspension, 0.5 ml of washed packed sheep red cells were added, incubated at 55°C water bath for 5 min, mixed, poured into a glass petridish and allowed to gel. Then, 3 mm diameter wells were punched, filled with 10 µl of different venom concentrations, incubated for at 37°C for 18-20h and the diameter of haemolytic haloes were measured. MIHD was calculated by plotting mean diameter of halo against venom concentration and reading off the dose corresponding to a 10 mm diameter.

SDS-PAGE electrophoresis

Vertical slab sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using discontinuous buffer system described by Laemmli [8]. A 12% separating and 4% stacking gel were used. 20 µl of reduced venom sample at a concentration of 1.5 mg/ml was used for loading sample through. Samples were electrophoresed at 200V for 45 min and gels were stained with 0.1% Commassie blue and destained in 7% acetic acid and 20% methanol until a clear back-ground was obtained. Gels were dried in gel dryer at 80°C for 2h.

Immunoblotting

Proteins were transferred from nonstained gels for immunoblotting onto a sheet of nitrocellulose paper following the method of Towbin et al. [9] using a Bio-Rad transblot apparatus. Immunostaining was carried out with primary antibody, antivenom raised in horse (commercial ASV) followed by peroxidase labelled anti-horse IgG.

RESULTS

Biological properties of venom

The results of the biological properties of venoms from 4 localities were shown in the table. Daik-U venom was highly lethal, haemor-

Table 1. Biological properties of venoms from 4 localities

Locality	Daik-U	Kungyan-kone	Htan-tabin	Htauk-kyant
Length of snake (cm)	81-120	101-110	80-100	90-120
No. of snake	8	10	3	6
LD_{50} i.v. µg/mouse	2.14	5.23	3.2	4.18
MHD µg/mouse	11.75	24.27	26.92	20.42
MND µg/rat	32.36	48.42	45.71	47.86
MDD µg/mouse	1.78	5.00	3.00	3.33
MCD µg/ml	6.4	5.1	16.5	19.4
MCPID µg	0.063	0.076	0.125	0.089
MIHD µg/ml	1.41	1.47	1.99	5.24

MHD = Minimum haemorrhagic dose
MND = Minimum necrotic dose
MDD = Minimum defibrinogenating dose
MCD = Minimum coagulant dose
MCPID = Minimum capillary permeability increasing dose
MIHD = Minimum indirect haemolytic dose

rhagic, necrotic and possessed potent defibrinogenating, CPI and phospholipase activities. Kungyankone venom was relatively less lethal, possessed poor defibrinogenating and potent coagulant and phospholipase activities compared to others. Htaukkyant and Htantabin venoms have similar properties except the former has poor phospholipase activity. Coagulant property of Daik-U and Kungyankone venoms was 2-3 times more potent than that of Htantabin and Htaukkyant.

SDS-PAGE electrophoresis

SDS-PAGE electrophoresis of venoms from different localities was shown in Fig. (2). There were quantitative differences in protein bands in between different localities. Venoms from Htaukkyant and Htantabin localities lacked 33-23KD and 21-16 KD bands. Immunoblotting of venoms from 4 localities showed quantitative differences in numbers of band Fig. (3).

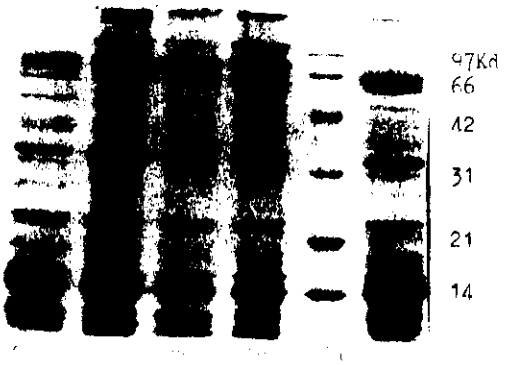

Fig. 2. SDS-PAGE electrophoresis of venoms from 4 localities using 12% separating gel. Lane 1-6 are Daik-U, Kungyankone, Htaukkyant, Htantabin, low M.W. markers and pooled reference venom respectively.

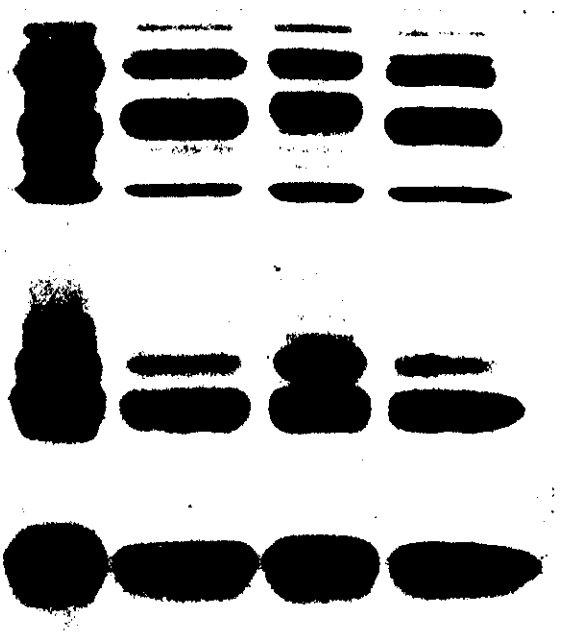

Fig. 3. Transblot nitrocellulose strip treated with horse antivenom and stained with peroxidase labelled rabbit antihorse IgG. lane 1 = pooled reference venom, lane 2 = Kungyankone lane 3 = Daik-U and lane 4 = Htantabin

DISCUSSION

Characterisation of biological properties of venom was carried out according to WHO recommended methods [1]. A preliminary study was conducted on venoms available from 4 localities. Further collection of venoms from different localities and characterisation of venoms are in progress. Since Russell's viper venom acts directly on factor X, procoagulant activity (MCD) of venom could not be demonstrated by MCD-P or MCD-F, instead plasma recalcification time was used to demonstrate its activity.

With regard to geographical variation of the biological properties of

venom, lethality and MDD of different localities tend to run in parallel. Venom from Daik-U locality was most lethal, haemorrhagic, necrotic and possessed potent MDD and phospholipase activities. Geographical variation in lethality was also observed in other species of snake [2-5]. No variation in MHD with regard to locality of rattlesnake was reported by Minton and Weinstein [3].

Although procoagulant activity of Htantabin/Htaukkyant was 3 times weaker than that of Kungyankone/Daik-U, the former possessed defibrinogenating activity. A similar observation was made by Theakston and Reid [1] on E. carinatus from Saudi Arabia which has no significant procoagulant activity, however it caused defibrinogenation in Vivo. Protein pattern of venom from a closed locality tends to have a similar pattern [10] which may explain similarity of protein pattern of venom from Htantabin and Htaukkyant.

Since MDD value of Russell's viper venom is near to or greater than LD_{50}, according to Reid and Theakston [11] defibrination in man can be considered to have serious clinical implication, however, caution is needed when extrapolating results of animal experiments to human. If defibrination occured in persons bitten by snake which has a trace of defibrinating activity, the prognosis would be extremely grave. It is likely that discrepancy in clinical pictures of snakebite patients from same or different localities was due to geographical variation of biological properties and composition of venom. Antivenom manufacturers should take into consideration of the finding in production of antivenom for common use. Poor neutralising activity of antivenoms was reported if the antivenom raised against venom from snakes of different geographical origin was used in different areas (Indian Haffkine antivenom used in Sri Lanka) [12].

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SHORT REPORT

Clinico-epidemiological factors of highly parasitised falciparum malaria patients

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Patients with over 10% parasitaemia is one of the signs of bad prognosis. Clinico-epidemiological study on such patients with high level of parasites in blood had not been reported.

The study was done from the records of the patients who attended the Tharyarwady Civil Hospital during 1986-89. The clinical charts of the patients with more than 10% of RBCs parasitised with falciparum malaria were collected for the study.

A total of 20 patients with over 10% of RBCs parasitised was recorded. Among them, there were 16 male patients, 4 were from malaria free area, Yangon. Out of them 8 patients expired, 5 expired within 24 hour of arrival to hospital and only two patients presented with unrousable coma.

Out of 4 female patients, 2 were with pregnancy. One with 3 month pregnancy, had mixed malaria infection with 82% parasitaemia level. She aborted and expired on the way to hospital. Second women had 6 month pregnancy and 31% of RBC were parasitised with mixed malaria infection. She was saved by Artemether injection and mefloquine tablet combination. They were semi-immune and were admitted at the same month from same elephant-training centre situated at the foot-hill of Bago Yoma.

There were three children among the patients. Two of them were from same village and admitted to the same hospital at the same week. The third child attended the hospital within an interval of 11 days with her mother who also belonged to the same group. There were 16 patients from the villages (semi-immune). Among them, 1 patient presented with unrousable coma. One of the semi-immune patient had history of cerebral malaria and had attended the same hospital 4 months ago. The parasitaemia varies among the patients, 8 of them had 20-82% and 12 had 10-20% parasitaemia level.

During the four years study from (1986-1989), there were 812 malaria patients among them these were the 20 patients with high level of parasitaemia in blood. The mortality rate in them was 40%. 62.5% of deaths occured within 24 hours of arrival to hospital. Standard antimalarials were not effective in these patients. So other form of treatment such as exchange blood transfusion as suggested by Hall and WHO [1,2] may be considered for these patients.

Two pregnant patients with mixed malaria infection had high level of parasites in blood. They had been staying in the same elephant-training village for a number of years.

Another two (mother & son) patients were from the same army agricultural centre. Another two children patients also belonged to the same village. Hence, this study suggested the existance of a special strain of P. falciparum malaria in some selected areas of the township.

The occurrance of cerebral malaria in the semi-immune patients may be another indicator to support the above hypothesis of having a special strain in the area.

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