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Are we spraying DDT at an appropriate period of time
to control malaria in Myanmar?

Myint-Htwe

Health Systems Research Unit,
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DDT spraying activity has been carried out to control malaria in Myanmar for many years. Appropriateness of spray timing is being evaluated by applying moving average method on slide positivity rates in five states and four divisions of the country. It was found that, in order to cover major transmission peaks in many areas, spray timing should be adjusted.

INTRODUCTION

Malaria has been identified as the most important public health problem in Myanmar. It has been accorded first priority disease in Third PHP (People's Health Program) (1986-1990) and first National Health Plan (NHP) (1991-1992). To achieve systematic implementation of malaria control operations, the country has been divided into five distinct strata taking into consideration a variety of determining factors for the occurrence of malaria [1]. Residual spraying is one of the major activities performed in stratum II (areas under control by spraying). These areas have been sprayed for many years and it is high time that spraying activities be reviewed in-depth in the context of correct spraying technique, logistics of spraying, timing of spraying and susceptibility status of insecticide used, etc. There has been little change in the spraying time for more than two decades. This study is an attempt to elucidate whether the current spraying time is appropriate or not in the context of present epidemiological conditions. The magnitude and importance of spraying activity in the country can be envisaged by observing the fact that

tons of DDT have been used yearly by the Vector Borne Diseases Control program [2].

The objectives of the study are

- (a) To elucidate the transmission season of malaria in different states and divisions.
- (b) To time the spraying activity in accord with the transmission period in respective states and divisions.

MATERIALS AND METHODS

It is a retrospective analysis of secondary data available at the Vector Borne Diseases Control Program, Headquarters, Yangon.

Study Area

States/Divisions which have complete sets of data on monthly slide positivity rates for at least 5 years (1984 to 1988) were taken [1]. A variety of different topographical areas (hilly, plain, coastal, forested) are included in the selected states and divisions. It was decided to analyze state and division-wise because factors governing the vector bionomics will be somewhat dissimilar in different

states and divisions. In other words, generalization for an area could not be made if overall seasonal variation of the country is examined in toto.

Study Variable

Slide positivity rates from sprayed areas have been chosen as the basic variable. The rationale for using it to determine transmission season is that the ratio of Plasmodium falciparum to Plasmodium vivax in different states and divisions from slide positive cases is in the region of 85:15 (1984-1987) [2]. There might be recrudescence of falciparum malaria but the numbers are few and far between. The distribution of blood slides examined covers all age groups and generally follows the population structure [1]. There is also no disproportionate examination of blood slides among various age groups [1]. Further elaboration of the data in terms of strata shows that there is no aggregation of falciparum malaria in one particular stratum or area, instead it is distributed somewhat evenly [1]. These facts further justify the use of slide positivity rate.

Study Methods

Transmission season can be determined by parasitological, entomological and epidemiological methods. The parasitological methods are more sensitive than entomological methods. Infant parasite rate is one of the sensitive indicators to determine transmission season e.g., longitudinal monthly infant parasite surveys starting with the new born infants. Use of morbidity statistics alone can be misleading because relapse of vivax malaria may occur when transmission is absent. When morbidity statistics are used, it is to be cautioned that seasonal rise starts 2 to 4 weeks after the first transmission has occurred. As in most of the infectious diseases, malaria exhibits

temporal variation which is characterized by seasonal variations, cyclical variations, secular trends and random variations [3]. Seasonal variations can be defined as changes in intensity or endemicity of malaria in a particular area at different times of the year. The fluctuation of a parameter area at different times of the year. The fluctuation of a parameter value (e.g., slide positivity rate) is contributed by the above four factors. In order to depict seasonal variation, the other three factors should be suppressed.

Classical decomposition analysis usually assumes a multiplicative relationship between the four components of a time series. Time series data, which are statistical data collected or observed at successive intervals of time, were used to depict the transmission season of malaria. Thus, a trend component is denoted by T , the cyclical component by C , the seasonal component by S and the irregular or random component by I . Each observation V of the time series can be expressed as
$$V_t = T_t * C_t * S_t * I_t$$
 at time period t .

If all variations in a time series were due entirely to seasonal influences, the specific seasonal for a given month is an index number where base is a mean monthly variate. The ratio-to-twelve-month moving average method relies on a monthly time series being affected by seasonal variations which repeat regularly year after year. The malaria data pattern can be correctly assumed as a seasonal time series data and thus computation of seasonal index is justified. Two reasons support the seasonal nature of malaria data.

- (a) The peaks and troughs are consistent over the years.
- (b) There is an explanation for the seasonal pattern.

Computation of Seasonal Index [3,4]

Seasonal Index is defined as the ratio of the actual value of the time series to the average for the year. There is a unique index for each period of the year. As the data are monthly data, it has 12 seasonal indices. A seasonal index of 126% in October is interpreted to mean that the number of cases (or parasite positive cases) in October is 126% of the number of cases in the average month. Similarly, an index of 65% in February is interpreted to mean that the number of cases in February is 65% of those in an average month. The following steps have been performed in order to obtain seasonal indices (Example computation is shown in Table 3).

(a) Computation of Centered 12 Month Moving Average

Computation of moving averages is based on the length of seasonality. As the seasonal index is aimed to get the monthly pattern of malaria transmission, 12 month moving totals were computed.

(b) Computation of Approximate Seasonal Index

The ratio of each month actual value to its centered 12 month moving average was computed. This is to eliminate or reduce the secular trend and cyclical variation from the data. Thus, it represents the seasonal or random component of the time series. These ratios are approximate seasonal indices.

(c) Refinement

The first refinement, in order to eliminate as much randomness as possible, was to compute the mean ratio for each month. All the ratios for a particular month were added and divided by the number of months involved.

(d) Computation of Normalizing Factor

Normalization factor was then computed using the formula, Normalizing Factor = $12/(\text{sum of mean ratios})$. This was done to adjust the mean ratios so that they summed to 12.

(e) Computation of Final Seasonal Indices (SI)

Ultimately, seasonal indices were calculated for each month by multiplying the mean ratio of each month by the normalization factor.

RESULTS AND DISCUSSION

Seasonal Indices for States

The following seasonal indices, based on slide positivity rates, were obtained for five states.

Table 1. Seasonal indices for five States

Month	Mon	Kayin	Kayah	Chin	Shan
Jan	*1.031	*1.199	0.826	0.954	0.671
Feb	0.937	0.951	0.464	1.081	0.637
Mar	0.908	0.973	0.464	0.831	0.744
Apr	0.812	1.122	0.671	0.885	0.642
May	0.751	0.939	1.034	*1.023	0.741
Jun	0.809	0.896	1.122	*1.216	0.974
Jul	0.950	0.864	1.105	0.982	*1.171
Aug	0.979	0.855	*1.154	0.847	*1.208
Sep	0.997	0.907	*1.195	*1.030	*1.121
Oct	*1.108	0.890	*1.241	*1.057	*1.512
Nov	*1.440	*1.283	*1.517	*1.126	*1.121
Dec	*1.341	*1.567	*1.449	0.898	*1.369

* presumed transmission season

State-wise Analysis on Seasonal Indices

Depending on the topography and vector prevalence of an area, transmission season will vary. The seasonal indices in Mon State appear to increase starting from the latter part of the year. This is also

supported by sporozoite dissection records in which highest positive rates were found in the latter part of the year (vector: An. dirus). The transmission season seems to be in the last quarter of the year. In Kayin State, two transmission peaks are observed, minor peak in April and major peak in November and December. The seasonal indices in Kayah State start to increase in June and reach its peak in November and December where 152% and 145% of the normal occurrences are respectively observed. Malaria transmission appears to be intense during the latter half of the year. The transmission season of malaria for this state is prolonged and covers almost three quarters of the year. In Chin State there are two peaks i.e., June and November. The transmission season appears to be shorter in this state compared to other states (vectors: An. minimus and An. maculatus). In Shan State, the transmission season seems to be in the latter half of the year (vector: An. minimus). The first half of the year has the lowest transmission potential.

Seasonal Indices for Divisions

The following seasonal indices, based on slide positivity rates, were obtained for four divisions.

Division-wise Analysis on Seasonal Indices

In Ayeyarwady Division, there are two peaks, one in April and the other one in November and December. Major transmission season appears to be in the last quarter of the year. In Mandalay Division there is only one transmission peak and spans a duration of five months. Yangon Division exhibits two peaks, minor one in April to June and the major one in November and December. Similar observations are noted in Bago Division. As per the data elucidated

Table 2. Seasonal Indices for Four Divisions

Month	Ayeyarwady	Mandalay	Yangon	Bago
Jan	0.989	0.504	*1.086	0.768
Feb	0.830	0.481	1.076	0.880
Mar	0.933	0.576	1.048	1.002
Apr	*1.021	0.611	*1.090	*1.131
May	*0.991	0.633	*1.085	*1.031
Jun	0.951	0.957	*1.127	*1.235
Jul	0.797	1.016	1.086	0.905
Aug	0.839	*1.177	0.329	0.808
Sep	0.848	*1.315	0.276	1.026
Oct	0.928	*1.432	0.442	*1.215
Nov	*1.297	*1.608	*1.090	*1.258
Dec	*1.621	*1.602	*1.748	*1.047

* presumed transmission season

in the tables mentioned above, it appears that some states and divisions have two transmission peaks.

Seasonal Pattern of Malaria in Myanmar

One of the factors which affects transmission season is ecological changes brought about by developmental activities which in turn lead to changes in vector distribution and vector bionomics especially longevity and biting rates. These changes together with various anti-malarial measures carried out over the years have significant impact on the transmission pattern of malaria. In the early eighties, transmission season of malaria usually coincides with the pre and post monsoon seasons. In some areas of Rakhine State, transmission is observed throughout the year. Practically speaking, transmission of malaria occurs in most areas throughout the year. But there is at least one major transmission peak in every state and division. At present, the density of primary vectors of malaria in Myanmar (An. minimus & An. dirus) is closely associated with seasonal pattern of malaria and hence

transmission season of malaria.

Table 3. Example computation of seasonal indices for Mon State

Slide positivity rates for Mon State from 1984 to 1988 are as follows:

Slide positivity rates % for Mon State, 1984-1988

Month	1984	1985	1986	1987	1988
Jan	7.92	8.79	9.76	9.70	9.08
Feb	4.62	7.27	6.28	12.20	8.30
Mar	7.52	6.96	4.95	10.20	11.20
Apr	8.27	5.98	4.18	8.75	9.37
May	7.28	5.13	5.05	7.99	9.37
Jun	8.67	7.40	5.00	7.50	9.77
Jul	9.02	9.76	5.83	9.05	9.53
Aug	10.40	10.10	5.68	8.86	8.35
Sep	9.61	11.50	6.50	8.19	8.75
Oct	8.65	12.70	8.89	9.44	13.40
Nov	16.50	14.90	10.20	10.60	14.20
Dec	15.20	11.40	10.60	11.50	13.70

12 month moving totals of SPR for Mon State

Month	1984	1985	1986	1987	1988
Jan		111.65	101.65	107.26	117.04
Feb		111.35	97.23	110.44	116.53
Mar		113.24	92.23	112.13	117.09
Apr		117.29	88.42	112.68	121.05
May		115.69	83.72	113.08	124.65
Jun	@113.66	111.89	82.92	113.98	126.85
Jul	*114.53	112.86	82.86	113.36	
Aug	117.18	111.87	88.78	109.46	
Sep	116.62	109.86	94.03	110.46	
Oct	114.33	108.06	98.60	112.91	
Nov	112.18	107.98	101.54	114.29	
Dec	110.91	105.58	104.04	116.56	

First 12 month moving total for 1984 =

$$1984 \text{ Jan SPR} + \dots + 1984 \text{ Dec SPR} \\ = (7.92 + \dots + 15.2) = @113.66$$

Second 12 month moving total for 1984 =

$$1984 \text{ Feb SPR} + \dots + 1985 \text{ Jan SPR} \\ = (4.62 + \dots + 8.79) = *114.53$$

Two twelve month moving total = 113.66 + 114.53 = 228.19

Approximate seasonal indices (SI) for Mon State.

Month	1984	1985	1986	1987	1988
Jan		0.947	1.130	1.101	0.932
Feb		0.782	0.757	1.344	0.852
Mar		0.743	0.627	1.099	1.150
Apr		0.622	0.555	0.934	1.128
May		0.528	0.704	0.849	0.915
Jun		0.780	0.720	0.792	0.932
Jul	0.948	1.042	0.844	0.955	
Aug	1.077	1.078	0.794	0.954	
Sep	0.986	1.244	0.853	0.893	
Oct	0.898	1.398	1.107	1.014	
Nov	1.748	1.655	1.223	1.119	
Dec	1.635	1.281	1.237	1.195	

Computation

12 month centered MA

$$= \frac{\Sigma \text{ first two 12 month moving totals}}{24} \quad (\text{for July})$$

$$= (228.19)/24$$

$$= 9.5079$$

Approximate SI (1984) July

$$= \frac{\text{July 1984 SPR}}{(\text{12 month centered MA for July})} \\ = 9.02/9.5079 \\ = 0.948$$

LEGEND. MA = Moving average

Mean Ratios (MR)

Mean ratios = Σ (Ratios for the respective month)/# of months

$$\text{MR for July} = (0.948 + 1.042 + 0.844 + 0.955)/4 = 0.947@$$

$$\text{MR for Aug} = (1.077 + 1.078 + 0.794 + 0.954)/4 = 0.976*$$

$$\text{MR for Sep} = (0.986 + 1.244 + 0.853 + 0.893)/4 = 0.944**$$

Normalization factor (NF)

NF = 12/(sum of mean ratios)

$$\text{NF} = 12/(1.028+0.934+0.905+\dots+1.337) = .99742$$

Final SI = Mean ratio for the month * NF

$$\text{Final SI for July} = \text{Mean ratio for July} * \text{NF} \\ = 0.947 * 0.99742 \\ = 0.95003$$

Mean ratios, normalizing factor and seasonal indices for Mon State.

Month	Mean Ratio	Norm. Factor	SI
Jan	1.028	0.99742	1.03087
Feb	0.934	0.99742	0.93694
Mar	0.905	0.99742	0.90766
Apr	0.810	0.99742	0.81228
May	0.749	0.99742	0.75124
Jun	0.806	0.99742	0.80848
Jul	0.947@	0.99742	0.95003
Aug	0.976**	0.99742	0.99716
Sep	0.994*	0.99742	0.99716
Oct	1.104	0.99742	1.10772
Nov	1.436	0.99742	1.44031
Dec	1.337	0.99742	1.34080

Discussion in the Context of Spraying

In most of the states/divisions under study, major transmission peak seems to be in the last quarter of the year. It may be due to specific and increasing problem of mobile population engaged in forest-related economy and gem mining. These people acquired malaria, transmitted by An. dirus and return to their respective townships whereby malaria is spread by either An. minimus or local vectors such as An. annularis, An. maculatus or An. sundaicus, etc. December enjoys the highest seasonal indices in most of the cases. Spraying activity was performed in all these areas from February to May. The main reason for conducting spraying activity during this period is because of favourable meteorological conditions and partly it might be due to the then transmission season (latter part of the seventies) when spraying activity was initiated. As the potency of DDT is wearing away during the latter part of its action span, it would result in more beneficial outcome if spraying could be performed during September and October i.e., it can completely cover the major transmission peak. Meteorological factors must be taken into

account in some areas from logistical point of view. The transmission and accessibility status of an area are also relatively good during this period. The transmission season determined by this method could be regarded as relatively crude in relation to immunological and vector studies. But in the context of the country's situation, it can be assumed as the most practicable approach to give insight into the appropriateness of spraying time. The seasonal index computed above is for the whole state/division. There might be some variation if it is determined township-wise.

Actually, an increase in slide positivity may not necessarily connote transmission season unless the slides are examined immediately after collection. Generally, the slides are examined one to two weeks after collection because these slides have to be sent to places where laboratory facilities and microscopists are available. But the positive results are registered according to the date of collection rather than the date of examination. Be that as it may, interpretation of this parameter should always be guarded because there are multitude of factors [5] which can modify the results of blood slide examination.

The elucidation of transmission season is not only for determination of spraying time but also for carrying out anti-malaria measures such as active surveillance, case treatment before this period. These can have dramatic influence on the size of case reservoir leading to reduction in transmission potential. One of the advantages of this method is that computation is relatively simple and can be easily taught to the peripheral staff so that s/he can monitor the situation well ahead of the crisis. In doing so, the staff will also appreciate the importance

of data and how useful information can be derived from the data at hand. This can enhance their positive attitude toward timely and systematic collection of data. A growing body of experience has shown that, once the health worker appreciates the usefulness of the data that they are collecting, it would pose no problem as to the acquisition of "quality" data. In coming to grips with the practical issues, a simple line graph depicting the seasonal indices can greatly help in the conduct of anti-malaria activities besides preparing in advance the expected increase in malaria situation in an area. One can also know whether the situation is actual increase or just seasonal variation of malaria in an area so that necessary activities can be performed. To be more effective, an area should be sprayed every 6 months as per the seasonal indices mentioned in tables (1) & (2). But in the context of the developing country's scenario, it is far from possible.

In conclusion, ideally, in keeping with the precepts of spraying, if one could spray before the major malaria transmission peak, it would exert more beneficial effect on the containment of malaria situation. The importance of spraying time could not be over emphasized. DDT spraying should be planned on the basis of ecological and epidemiological stratification. The problem of vector resistance, reduced biological efficacy of the sprayed surfaces, reduced compliance by the community of household spraying, etc. should be considered in spray planning. In essence, the above findings clearly indicate that time of spraying should be adjusted according to the transmission season of the area concerned. Assuming that the logistics of spraying is going on smoothly and proper training has been given to the spray men, the only

thing that matters most is time of spraying. If one can spray DDT at an appropriate time, one is bound to meet with success. It can therefore be stated that current time of spraying is inappropriate given the fact that transmission peaks are in the fourth quarters of every year in almost all the areas under study. In line with the current concept of malaria control, it is not justified to use major proportion of malaria budget for spraying. Impregnated (pyrethroid) bed nets and curtains are better alternative to DDT spraying. It is needless to emphasize that integrated vector control methods should be practiced wherever feasible. Be that as it may, township-wise in-depth analysis, including vector bionomics, is strongly recommended before radically switching the spraying time in respective areas.

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Haematological features of falciparum malaria patients
with anaemia at Mandalay General Hospital

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Fifty falciparum malaria patients who attended consecutively to Mandalay General Hospital for anaemia were studied. Of these, 26% had haemoglobin less than 5 gm %, 78% had hypochromic microcytic anaemia, 16% was found with hypochromic normocytic anaemia and 6% showed normochromic normocytic anaemia picture. They all were free from G6PD deficiency, hookworm in stool, and their Coombs test (direct and indirect) were negative. Enlargement of liver and spleen was recorded in 84% and 64% of patients respectively. Mean $\pm$ SD serum bilirubin was 11.9 ± 5.4 μ mol/l. Patients with increased serum bilirubin was recorded in only 6% of the patients. Mean conjugated bilirubin level was 8.6 ± 5.2 μ mol/l. There was no predominance of conjugated bilirubin in patients with raised serum bilirubin. There was no correlation between parasite density and total serum bilirubin or haemoglobin level of the blood. Mean reticulocyte count of the patients was 1.1/cmm. Reticulocyte count above 2% was recorded in only 8% of patients. The mean serum iron level was 83.75 ± 20.7 μ g/100 ml (82.7 μ g/100 ml for males and 85.5 μ g/100 ml for females). The lowest serum iron level was 40 μ g/100 ml. Bone marrow showed normoblastic picture in 62%, megaloblastic picture in 8%, dimorphic (megaloblastic and normoblastic) in 8% and erythroid hyperplasia in 22% of the patients. Iron content in bone marrow was normal in 72% and reduced in 28% of cases. Bone marrow with no stainable iron was not recorded in this study. Blood transfusion may be the only immediate form of treatment of patients with severe anaemia (PCV less than 29%) in case of emergency.

INTRODUCTION

Anaemia is an important complication in patients with acute and chronic *P. falciparum* malaria in endemic areas, and it is a less serious problem in other human malarial infections [1]. As there were very few clinical studies on malaria with anaemia, a study was therefore undertaken to determine the haematological parameters of anaemia falciparum malaria in-patients.

PATIENTS AND METHODS

Fifty adult patients with *Plasmodium falciparum* malaria with anaemia (Hb less than 13 gm % in males and less than 12 gm % in females) who were admitted to three medical units of Mandalay General Hospital were included in the study.

Patients with mixed infection, cerebral signs and symptoms, bleeding

piles, peptic ulcer, hook worm infestation and other associated chronic infections and children under 12 years of age were excluded from the study.

Blood was taken from the patients with anaemia to examine the following:

- (1) Thin blood film for the presence of malaria parasites and thick film for parasite density which was graded as 1 to 4 + according to Burce-Chwatt scale [2].
- (2) Hb %, total and differential count of WBC and reticulocyte counts.
- (3) Serum iron and bilirubin (total and conjugated), G6PD and Coombs test.
- (4) Urine for urobilinogen and haemoglobinuria.

In addition, bone marrow aspiration was taken from the posterior iliac crest of the patients to determine bone marrow iron. Routine laboratory methods as described by Dacie & Lewis [3] were used for the tests at the Haematology Department of the Mandalay General Hospital.

RESULTS

Altogether, 50 patients with Plasmodium falciparum infection with anaemia were studied. Of these, 58% were males and 42% were females. Their ages ranged from 12 to 67 years and the average age was 25.6 years. 26% of patients (24.2% of males and 28.6% of females) had haemoglobin less than 5 gm %. There was no correlation between the parasite density and haemoglobin level.

Most (86%) of the patients showed normal reticulocyte count and the average count was 1.1%. There was no correlation between parasite density and reticulocyte count. With respect to anaemia 78% had hypochromic

microcytic, 16% had hypochromic normocytic and 6% had normochromic normocytic blood picture.

The mean $\pm$ SD serum iron level was 83.7 ± 20.7 ug/100 ml (82.7 ug/100 ml for males and 85.5 ug/100 ml for females). The lowest serum iron level was 40 ug/100 ml. There was no correlation between the parasite density and serum iron level.

The mean $\pm$ SD serum bilirubin level of the patients was 11.9 ± 5.4 umol/l. There was no correlation between the parasite density and serum bilirubin level of the patients. The mean conjugated serum bilirubin of the patients was 8.6 ± 5.2 umol/l and only 6% of the patients had increased serum bilirubin above 21 umol/l.

72% of patients had normal iron staining and 28% had reduced iron staining in bone marrow. Patients with total iron depletion in bone marrow were not observed. Three types of bone marrow films were recorded. 62% had normal bone marrow picture and normal myeloid erythroid ratio, 22% showed erythroid hyperplasia, 16% had megloblastic changes (8% purely megloblastic, 8% dimorphic). Patients with bone marrow depletion and dyserythropoiesis were not observed. Malaria parasites were detected in bone marrow of 12 patients (24%). Gametocytes were present in bone marrow of 4 patients (8%).

6% of patients had jaundice. The analysis of their serum bilirubin (direct and indirect), SGPT, urine urobilinogen revealed the nature of jaundice as haemolytic with essentially normal SGPT values.

All the patients had no G6PD deficiency and direct and indirect Coombs Test were negative. 84%

showed enlargement of liver and 64% had splenic enlargement.

DISCUSSION

In this study, among the anaemic patients, about a quarter of males or females had severe anaemia of less than 5 gm %. In one study carried out at Tharyarwady Civil Hospital, 18% of the malaria patients had PCV less than 20% on admission. Hence, anaemia is a common problem in falciparum malaria cases.

In this study, 6% of patients had evidence of haemolysis. Tin Shwe et al [4] had also found low level of serum hepatoglobin, patients with ahepatoglobinemia was not observed among malaria patients. In this study, there was no correlation among the haemoglobin level, serum bilirubin and parasite load of the patients. Most of the patients had normal bone marrow picture. Among the patients with raised serum bilirubin, there was no predominance of conjugated bilirubin. Hence the mechanism of anaemia in malaria may be multifactorial and not due to haemolysis alone.

In this study, serum iron was reduced in 26% of patients. Such reduction in the serum iron level is common in patients with infections.

The finding of normal iron store in bone marrow, among the 8 patients with reduced serum iron level (less than 80 ug) indicated that depletion of iron store may not be the cause of anaemia and iron supplementation therapy may not be required for the correction of anaemia in malaria cases.

In normal subjects the reticulocyte count varies from 0.2 to 2%. In haemolytic anaemia it usually ranges

from 5 to 20% but occasionally rises to much higher values, e.g. from 50 to 70% or even more [6]. In this study, the mean reticulocyte count of the patients was 1.1%. The highest reticulocyte count recorded was 6.8%. Hence there may be some delay in formation of new RBCs from bone marrow.

In our other study, some of the malaria patients had further reduction in haemoglobin level after the parasites had disappeared from the circulation [1]. Red cell survival studied carried out on malaria patients a few days after clearance of parasites from the circulation showed significant reduction in the life span of RBCs [7].

Consideration of falciparum malaria cases with microcytic hypochromic anaemia with normal iron store and low reticulocyte count, blood transfusion may be the only answer for the immediate management of such cases.

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Plasmodium falciparum; merozoite invasion rate in
erythrocytes with β -thalassaemia trait and
glucose-6-phosphate dehydrogenase
deficient genes

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The malaria parasite invasion-erythrocyte membrane protein variation hypothesis was tested on red cells collected from subjects with G-6-PD deficiency and β -thalassaemia trait genes. A series of *in vitro* competition assays were carried out by using serial dilution technique and the FITC-labelling method [1]. The results showed that the invasion rates of P. falciparum merozoites into two G-6-PD hemizygotes deficient cell were Gd^B- 100% of normal and Gd-Myanmar 95% of normal. But in erythrocytes from two patients with double gene defects viz. G-6-PD mild deficient gene Gd^B- with β -thalassaemia trait gene was 25% and G-6-PD severely deficient gene Gd-Myanmar with β -thalassaemia trait gene was 20% susceptible to merozoite invasion. The invasion rate of malaria merozoites in these cells could be inhibited by the partial loss of vital attachment of the red cell membrane required for the process of invasion.

INTRODUCTION

Recent progress in defining the human erythrocyte receptors for Plasmodium falciparum was reported by Pasvol [2] who presented results which suggested that two different major erythrocyte membrane glycoproteins are involved in the invasion by P. falciparum merozoites. The glycoproteins involved in invasion process are α and γ -glycophorins which binds non-covalently to the main transmembrane protein, band 3, a portion being linked to the cytoskeletal web on the cytoplasmic surface of the erythrocyte by ankyrin (band 2.1). Friedman *et al.* [3] and Okoye & Bennet [4] also reported evidence that the oligosaccharide portion of band 3 (erythroglycan) is a primary interaction site for P. falciparum merozoites.

A few studies have been concerned with invasion by malarial parasites to red cells which have modified cytoskeletons. These include investigation of erythrocytes lacking glycophorin or with increased rigidity such as sickle cells [5,6] cells from homozygous hemoglobin C [7] and melanesian ovalocytes [8,9] all of which resistance to invasion by malarial parasite.

Identification of molecular structure of membrane components and cytoskeletal protein patterns of red cells with various genetic defects in relation to malaria parasite invasion should shed light on the mode of attachments of malarial merozoites to these abnormal erythrocytes. We describe here a preliminary study with two common genetic defects of red cells G-6-PD

deficiency and β -thalassaemia trait genes which are highly prevalent in Myanmar.

MATERIALS AND METHODS

Venous blood in ACD (Acid-Citrate-dextrose, 1:4) was collected from random subjects coming to (i) the Blood Bank, Yangon General Hospital and (ii) the Defense Services General Hospital, Mingaladon. PIGPA treatment (50 mM pyruvate, 50 mM sodium chloride, 5 mM adenine) was carried out with all red cells in order to replenish ATP level [10].

Laboratory confirmation of the presence of thalassaemia trait genes, G-6-PD deficiency and haemoglobin variants was done according to the standard methods [11,12,13].

Cultivation, synchronization and harvesting of *P. falciparum* isolates (DMR/KHA-005) late schizonts was carried out as described by Trager & Jensen [14], Lumbros & Vanderberg [15], and Dluzewski *et al.* [16] using 78% Percoll with gradient centrifugation at 1800 rpm Kg for 15 minutes. (Fig. 1) a photomicrograph of harvested schizonts of *P. falciparum* from continuous culture. The same isolate (DMR/KHA 005) was used throughout the experiments. The schizonts were well synchronized and mature, contamination with red cells was less than 5%.

Invasion assays were performed by FITC (fluorescence isothiocyanate) labelling method of Lamont *et al.* [1]. The serial dilution technique used is outlined in flow chart given in (Fig. 2).

RESULTS

Out of the 373 subjects screened, 18

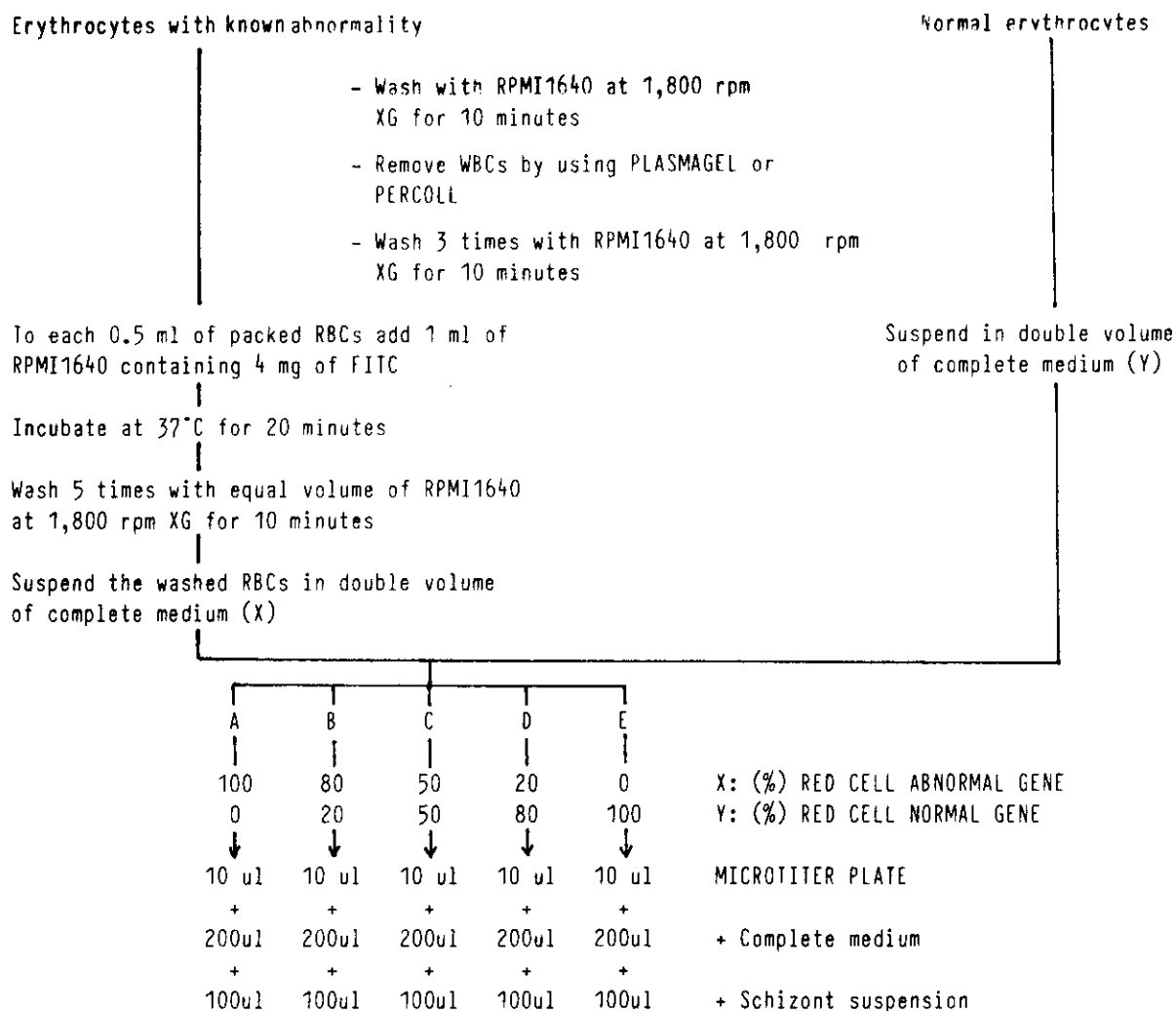


Fig. 1. Harvested late schizonts forms of *Plasmodium falciparum* isolate (DMR/KHA-005)

subjects with G-6-PD deficient (Gd^{B-}) hemizygous gene, 16 subjects with G-6-PD deficient (Gd -Myanmar) hemizygous gene, 40 subjects with β -thalassaemia trait and 20 subjects with double gene defects were selected for the study.

Among the (20) double gene defected subjects, 11 subjects were G-6-PD deficient (Gd^{B-}) hemizygous gene with β -thalassaemia trait gene, 9 subjects were G-6-PD deficient Gd -Myanmar hemizygous gene with β -thalassaemia trait gene.

The *falciparum* merozoite invasion assays were carried out and completed on (a) 12 subjects with G-6-PD (Gd^{B-}), (b) 11 subjects with G-6-PD (Gd -Myanmar), (c) 12 subjects with β -thalassaemia trait (d) 10 subjects with G-6-PD (Gd^{B-}) and β -thalassaemia and (e) 8 subjects



- (1) Incubate overnight
- (2) Take triplicate thin smears from each well
- (3) Fix in methanol and stain with 0.2% Ethidium Bromide
- (4) Count ring trophozoites in FITC labelled cells (10^3) and unlabelled cells (10^3)

Fig. 2. Plasmodium falciparum merozoites invasion assays (work plan)

with G-6-PD (Gd-Myanmar) and β -thalassaemia genes.

Figure 3 represents the merozoite invasion rate of P. falciparum to various types of erythrocytes. Both types of G-6-PD deficient red cell (Gd^{B-} and Gd-Myanmar) and red cell with β -thalassaemia trait gene at various dilution show 100%, 95% and 98% invasion rates compared to

normal red cells.

But red cells with double gene objects i.e. (both G-6-PD deficient and β -thalassaemia trait genes) were not completely susceptible to P. falciparum merozoites invasion. Only (25%) invasion rate with Gd^{B-} as well as β -thalassaemia trait red cells and only 1/3 (20%) invasion rate with Gd-Myanmar as well as

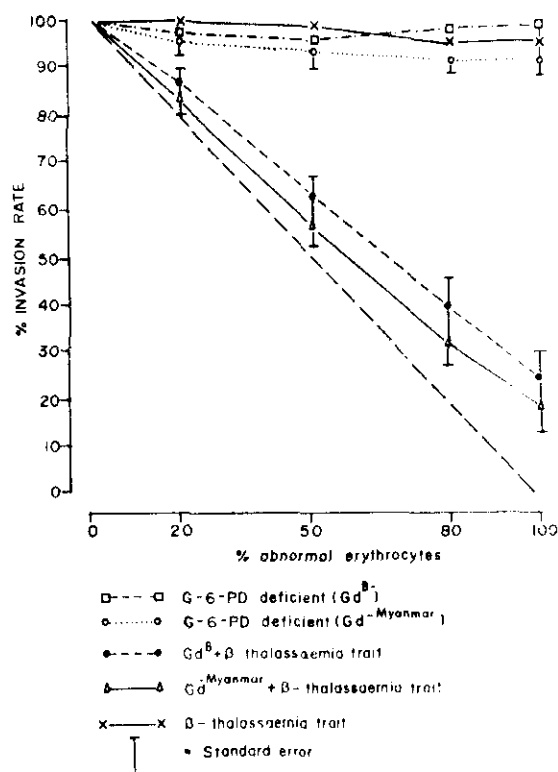


Fig. 3. Invasion rates of *Plasmodium falciparum* merozoites into genetically abnormal erythrocytes

β -thalassaemia double gene defected red cells were recorded at 100% of abnormal red cells in culture.

DISCUSSION

An important phase in the proliferation of the malarial parasite is the invasion of the red cell by the merozoite which leads to its multiplication within the erythrocytes and the eventual rupture of the cell and release of daughter merozoites. The invasion of the erythrocyte by the malarial parasite involves both interaction with an erythrocyte surface receptor and the disruption, or dissociation from membrane of the erythrocyte

cytoskeleton [17]. The importance of the cytoskeleton in red cell viability is emphasized by increasing evidence that several types of hemolytic anaemia (which usually involve mishapen erythrocytes) are associated with different molecular defects in the cytoskeletal system [18].

The specific defect of the spectrin molecule in hereditary spherocytosis (HS), elliptocytosis (HE), and pyropoikilocytosis (HPP) have long been suspected and several reports on spectrin abnormalities, particularly in relation to heat stability have been reported [19]. The membrane proteins of the G-6-PD deficient red cells were first analysed on SDS-PAGE (Sodium dodecyl sulphate Polyacrylamide gel electrophoresis) by AFOLAYAN [20] and by JOHNSON *et al.* [21], who also found that red cells with four different variants associated with unusual high molecular polypeptide aggregates which consist of spectrin and other protein but not globin.

It has been reported the membrane proteins of G-6-PD deficient red cells were normal, whereas membrane proteins from red cells with double gene defects were found to be partially reduced in spectrin and band 3, with a significant deficiency of glycophorin A [22].

In our study, we found an evidence of decreased invasion rates of *P. falciparum* merozoites into abnormal erythrocytes with double genetic defects (either G-6-PD mild deficient gene, Gd^{B-} or G-6-PD severe deficient gene, Gd^{Myanmar} with β -thalassaemia trait genes). It could be emphasized that the events of the invasion process by *P. falciparum* merozoites into these cells at the structural and biochemical levels remains to be

elucidated. In 1990, Wilson [23] postulated that the hypothetical scheme is the arrangements of red cell membrane proteins which culminates in invasion.

Therefore, our results reveals that the attachment of *P. falciparum* merozoites to red cells with double gene defect could be interrupted by the insufficient amount of vital attachments on these erythrocyte membranes.

However, it must be emphasized that the conditions of *in vitro* cultivation can be no means be considered physiological.. It is desirable to carry out more extensive studies on the relationship of red cell disorders and severity of malaria infection in hospitalized patients.

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A rapid diagnosis of malaria parasites by acridine
orange staining method

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The study was designed to find out the diagnostic value of Acridine orange staining method in detection of malaria parasite (Plasmodium falciparum) in comparison with the conventional thick and thin blood films. Thirty two P. falciparum and two P. vivax malaria cases were included in the study and the thick blood film and acridine orange staining methods were found to be more sensitive in detecting asexual and sexual parasites (gametocytes) than the thin film. It needs 2-4 weeks time to train a technician to be able to detect the parasites in thick and thin film methods, but acridine orange staining method can be transferred within an hour. Another advantage of acridine orange staining method is the shortest examination time, lasting only 45 seconds whereas the thick and thin blood films need 5 to 10 minutes. Hence, it is concluded that the acridine orange staining method is useful for quick diagnosis of malaria although accessory equipment is required.

INTRODUCTION

Current malaria diagnosis is done by microscopic reading of Giemsa stained thick and thin smears. This requires trained technicians who can process about 40-60 samples a day. The process is both time consuming and variable with individual reader.

Though the DNA or RNA probe methods allow batchwise sampling and analysis eliminates many of the problems associated with individual reader bias. The methods are not feasible for the field study.

A fluorochrome method is available for the rapid detection of low densities of malaria parasites in peripheral blood films. This method depends on the high-intensity fluorescence of malaria parasites in

thick blood films stained with acridine orange. This intensity allows the use of low magnification and permits rapid scanning of large areas. Specific diagnosis can be confirmed by subsequent staining of the same blood films with a conventional Giemsa stain. The study is to determine the applicability and sensitivity of acridine orange staining method in comparison with that of the standard thick and thin blood film staining methods in the identification of malaria parasites.

PATIENTS AND METHODS

(a) Subjects and area of study

The malaria patients were selected from clinically suspected cases of malaria admitted to Defence Services

General Hospital, Mingaladon.

(b) Preparation and staining of blood films

Thick and thin blood films were made from venous blood on the same microscopic glass slide (6 x 25 mm). The films were stained with 10% Giemsa solution.

(c) Microscopic examination of malaria parasites

The parasites were examined under paired x 10 oculars and an x 100 oil immersion lens with evenly distributed RBC (about 200 red blood cells per thin field) and an area of WBC 7-10 per oil field was selected in the thick films.

(d) Preparation and staining with Acridine orange staining solution

Acridine orange stock solution in distilled water (10g/L) is allowed to mature for 4 weeks before use. 250 ul of the stock solution was then diluted with 10 ml of Sorensen's phosphate buffer solution (pH 6.4). The solution was then used only for 2 or 3 days and discarded after a week. After haemolysing the thick film in distilled water for 2 minutes, the slide was immersed in a jar of acridine orange solution for 8-10 seconds. The slide was then dipped briefly in two changes of distilled water, left soaking in a jar of distilled water (or local tap water) for 3 minutes, and covered with a cover slip.

(e) Examination by fluorescence microscope

An Olympus fluorescence microscope Model BH was used with X10 wide angle ocular lenses, achromatic X40 objective, a dark ground condenser and mercury arc lamp using exciting filter BG12 and barrier filter 0 515.

(f) Calculation of conversion factor

(i) Thin film

Parasite counts in the thin films have been expressed in terms of 1000 field examination.

The volume of 1000 fields of a thin film as 25.45 mm (average thickness) x 0.0025 mm² or 0.0636 mm³. To convert an area count per 1000 fields of a thin film to a volume-tric count per mm³, the count is multiplied by 1/0.0636, i.e. by 15.7.

(ii) Thick film

Parasite counts in the series were recorded per 100 fields of thick film. The volume of 100 fields of average thickness of a thick film is 2.545 x 0.63 mm (average thickness) or 0.16 mm³.

To convert a count per 100 fields into a count per mm³, the parasite count was multiplied by 1/0.227, i.e. by 4.4.

RESULTS AND DISCUSSION

The experiment was conducted on 56 clinically suspected malaria patients. The efficacy of the three methods of examination of parasite was carried out on 34 malaria positive cases (thick film 100 fields). The lowest number of slide positivity (95.3%), species identification rate (90.6%) and gametocyte identification rate (41.7%) were noted in thin film method against the other two methods (Fig. 1). The result is comparable with that of a study performed in Nigeria showing that the thick blood film method was 1.4 times more sensitive in detecting trophozoites, and 8 times more sensitive in detecting gametocytes of *Plasmodium falciparum* than the thin film (Storey *et al.*,

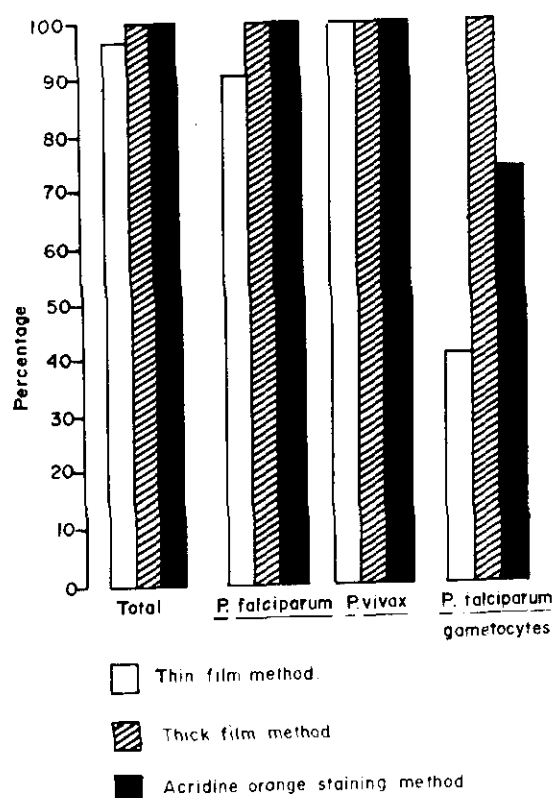


Fig. 1. Histograms showing comparative efficiency of thin, thick and acridine orange staining methods of blood film examinations of positive cases only

1973) [3].

Both the thick blood film examination method and the acridine orange staining method, gave the identical slide positivity rate of 100% and the number of species identification rate of 100%. However the gametocyte identification rate was higher in the thick film method (100%) and lower in the acridine orange staining method (75%).

The comparison of thick and thin film methods were carried out with different level of parasitaemia (counted by 100 fields thick film). The thick film method was shown to

be more efficient in detecting low level parasitaemia (less than 100 per 100 fields) than the thin film (Table 1). However the thin film method was found to be superior in detecting high level parasitaemia than the thick film. The corrected parasite count was higher in the thin film method. The corrected parasite count for the thin films was 1.7 times that for the thick films (Table 1). Dowling & Shute (1966) express that the corrected parasite count for the thin film series was, 2.5 times that for the thick films [1].

The relationship of the total slide positivity rates of the thick film and acridine orange staining methods of blood film were shown in Fig. 2. According to Spearman's Rank Sum Correlation Test, they are significantly correlated, which indicates that the acridine orange staining method is as reliable as the thick blood film examination method for the diagnosis of malaria.

The thick smear contains 20 times the amount of blood than the thin smear, so one can examine more volume of blood at the same time. The results of the present investigation suggested that a high proportion of parasites (39.76%) were lost during staining. In a heavy infection this parasites loss is of little importance, but in case of low parasitaemia, the loss could be of greater significance.

Dowling and Shute (1966) also showed the corrected counts of 65% loss of parasites in the thick film as compared with the corresponding thin film, which occurred during the dehaemoglobinization and staining process. It is found that acridine orange staining method and thick film method are more sensitive in identification of gametocytes than the thin film examination method.

Table 1. Comparison of corrected total parasite counts in 34 positive thick and thin films, showing variation with degree of parasitaemia

Degree of parasitaemia	Type of film	No. Examined	Observed No. of parasite per film	Corrected parasite count per mm ³	Ratio of average count (thin : thick)
Low (less than 100 per 100 fields of thick film)	Thick	5	65	286	0
	Thin	5	0	0	
Medium (100-999 per 100 fields of thick film)	Thick	6	329.17	1448.35	2.09
	Thin	6	193.33	3035.33	
Heavy (1000-9999 per 100 fields of thick film)	Thick	16	3714.38	16343.27	2.3
	Thin	16	2397.5	37640.75	
Very heavy (10000 & over per 100 fields of thick film)	Thick	7	24957.14	109811.42	1.43
	Thin	7	10005.71	157089.71	
Total (all classes of parasitaemia)	Thick	34	6948.97	30575.47	1.7
	Thin	34	3230.59	50720.26	

per 100 fields for thick film and per 1000 field for thin film

This may be due to lower density of gametocytes in the blood. Acridine orange staining method is more rapid in detection of malaria parasites than routine examination of thick and thin blood films. According to Sodeman & Jeffery (1968) the fluorochrome stain method is more efficient in evaluation of the malaria control programmes and is useful in identifying malaria carriers among blood donors [2].

The advantages of acridine orange staining method include less examination time (i.e. 45 seconds) because the fluorescent intensity allows the use of low magnification and permit rapid scanning of large areas and need only short training. The requirement of a fluorescence

microscope and the presence of fluorescing artefacts are the two disadvantages.

The three different methods showed no significant differences in sensitivity of diagnosis of malaria parasites. However time consumption is lowest with acridine orange staining method and highest with the thin blood film examination method. Therefore the acridine orange staining method is useful for detection of malaria parasite in urgent situation for treatment of malaria patients. Hence, the acridine orange staining method can be a life saving procedure for a malaria patient who suffered from severe complications of malaria.

[Spearman's rank sum correlation test value $r_s = 0.8309$
significant at 5% level (5% = 0.361)]

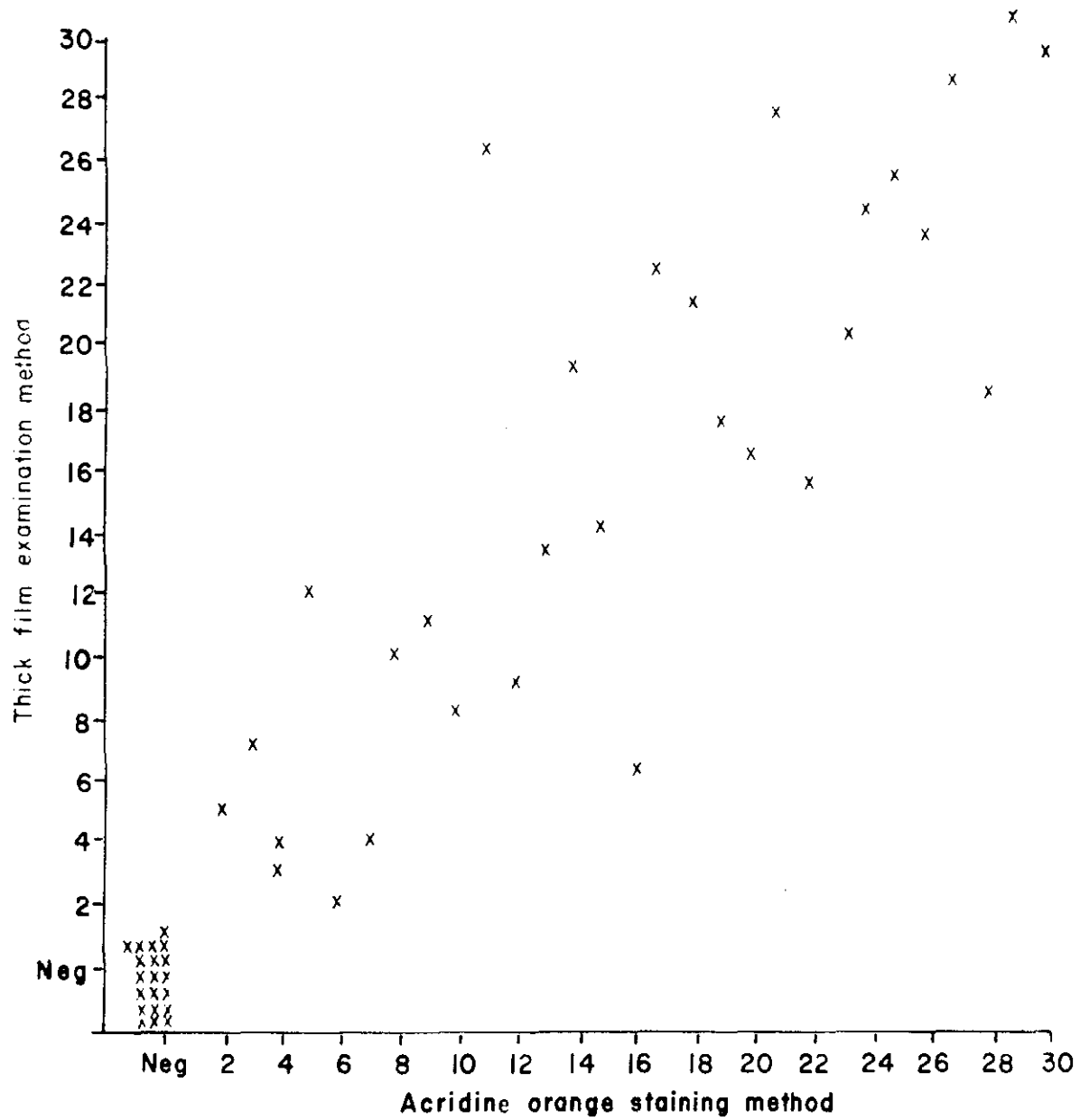


Fig.2. The relationship between rank order for parasite count per mm of thick film examination method and acridine orange staining method

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Study of inhibitory effects of some traditional medicine
formulations on adrenaline-induced hyperglycaemia
in animal models

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Traditional medicine formulations TMF-27, TMF-28 and TMF-32 have been used traditionally to treat diabetes in Myanmar. Blood glucose levels of adrenaline-induced diabetic rabbits were determined after oral administration of maximum dose of the traditional medicine formulations TMF-27, TMF-28 and TMF-32 and their watery extracts respectively. From the data obtained, it was concluded that the oral administration of 1.6 g/kg of TMFs did not produce inhibition of hyperglycaemic effect in rabbits. Only the water soluble extract of TMF-32 inhibited the hyperglycaemic blood glucose level on adrenaline-diabetic rabbits ($p < 0.05$) and rats ($p < 0.05$).

INTRODUCTION

Traditional medicine formulations have been used for the treatment of diabetes, yet the scientific data seems to be lacking to support its value in the disease.

Farnsworth [1] 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 preparations or as pure active principles". Many plants have been used throughout the world for the treatment of diabetes mellitus.

Mya-Bwin & Sein-Gwan [2] and Oliver [3] mentioned a large number of herbal drugs which are reputed for the treatment of diabetes mellitus.

Myanmar is rich in plants many of which are of medicinal values. A search for effective treatment of this common disease by products of

indigenous plants or traditional medicine formulations has been desirable, both from an academic as well as an economic point of view, in consideration of availability and accessibility of drugs locally. But, so far, there has not been full and systematic exploitation of these natural resources with regard to hypoglycaemic effect. Thus, in this investigation are presented the hypoglycaemic effects of watery extract of the traditional medicine formulations such as TMF-27, TMF-28 and TMF-32 which were claimed to have therapeutic values in the treatment of diabetes.

MATERIALS AND METHODS

(a) Preparation of the extract

Crude watery extracts were prepared by refluxing 100 g of each TMF powder in a round bottomed flask on a boiling water bath using 400 ml of water for 12 hours. It was centrifuged and the filtrate was evapo-

rated to dryness on a boiling water bath. The extract obtained was suspended in 1% methyl cellulose before administration to respective animals.

(1) Inhibitory effect of TMFs and TMFs watery extracts on adrenaline-induced hyperglycaemia in rabbits and rats

Adult healthy rabbits of JB strain weighing 2.33 ± 0.12 kg were used in these experiments. A group of rabbits were made diabetic by injecting them subcutaneously with 10 mg/kg of adrenaline tartrate using the method of Gupta, Verma, Garg & Mahesh Rai [4]. The rabbits were randomly divided into 8 different groups of 3 animals each. They were fasted overnight, at which time (0 hr) blood samples taken from the marginal ear-vein and deproteinized in trichloroacetic acid to prevent glycolysis. It was then immediately followed by the oral administration of either the test drug or tolbutamide or the placebo. Group I served as an untreated control as they received orally 10 ml of vehicle only. The animals of group II - IV were treated orally with 1.6 g/kg body weight of TMFs powders suspended in 10 ml of 1% methyl cellulose. The animals of groups V - VII received 2.3 g of the watery extracts of the TMFs equivalent to 6.4 g/kg body weight of the powders orally. The amount of TMFs or their aqueous extracts required for each rabbit was calculated on body weight basis and that amount of drug was weighed, well triturated with vehicle and then administered orally to each animal by using a feeding nalgene tube. Group VIII was treated with 120 mg/kg of tolbutamide in 1% methyl cellulose. After drug administration, serial blood samples were taken at 1, 2 and 4 hr respectively. Blood glucose levels of the samples were deter-

mined by the published o-toluidine method [5].

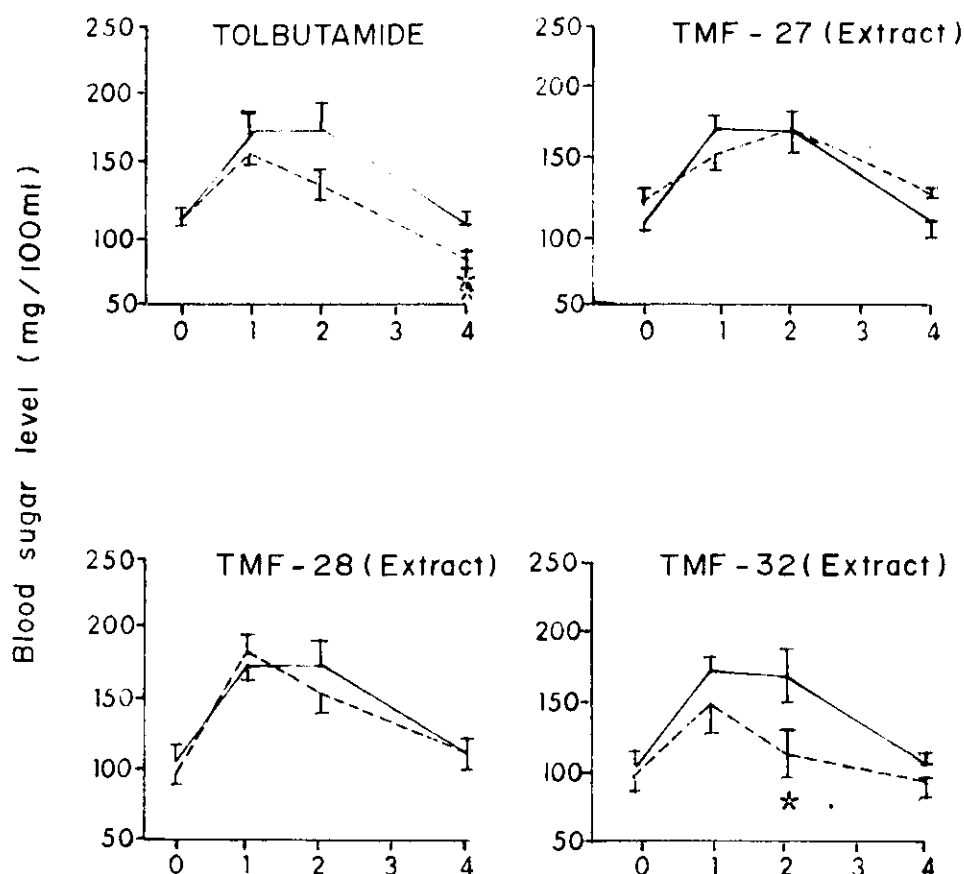
A cross-over test between the groups was repeated after one week's interval of rest. Time-course effect of the test drugs were compared to that of the classical drug tolbutamide, and the hypoglycaemic activity analysed statistically.

A similar investigation using adrenaline-induced hyperglycaemia was also conducted on adult male albino-rats weighing 245 ± 3.7 g. The only difference was that the experiment was carried out only with watery extract of TMF-32 and standard drug, tolbutamide. The blood glucose levels of the samples were determined as previously described method.

RESULTS

Effect of TMFs powder and watery extracts on blood glucose levels of diabetic rabbits and rats

The injection of adrenaline significantly induced hyperglycaemia ($p < 0.005$) in the rabbits reaching its maximum in 1-2 hr. The standard test-agent tolbutamide significantly inhibited the blood glucose level when comparison was made between the test and control groups at 4 hr ($p < 0.005$). Administration of 1.6 g/kg of TMFs powders produced a non-significant inhibition in blood glucose throughout the 4 hr trial period when compared with that of the control group. However, rabbits treated with 2.3 g/kg of TMF-32 watery extract which was equivalent to 6.4 g/kg of TMF powder showed significant ($p < 0.05$) inhibition at 2 hr as shown in Fig. 1. The plant ingredients contained in TMF-32 were listed as shown in Table 1. Similarly, the standard positive control



TIME AFTER DRUG ADMINISTRATION AND ADRENALINE INDUCTION(hr)

Fig. 1. Time course of the effects of TMFs watery extracts and standard drug on adrenaline-induced hyperglycemic rabbit model.

Each point represents the mean of observations and vertical bar indicates standard error of the mean. The groups tested were --, placebo, (methyl cellulose) and ---, the test drugs respectively.

* denotes $p < 0.05$

** denotes $p < 0.005$

tolbutamide significantly inhibited the blood glucose level in rats at 2 hr ($p < 0.025$) and 4 hr ($p < 0.005$) as shown in Fig. 2. Moreover, 2.3 g/kg of TMF-32 watery extract equivalent to 6.4 g/kg of TMF powder also produced significant inhibition in blood glucose level at 2 hr ($p < 0.05$).

DISCUSSION

In the present investigation, one dose of each TMF powder and TMF watery extract on blood sugar were assayed against the hyperglycemic response of adrenaline in rabbits and rats. Analysis of the data presented in the figures revealed

Table 1. Ingredients of IMF-32 (Nandwin hsabut hsay gye)

Description of item				
Scientific name	Family	Myanmar name	Part used	Amount in %
1. <i>Valeriana wallichii</i> DC.	Valerianaceae	Kantbalu	Rhizome	4.2
2. <i>Kaempferia</i> sp.	Zingiberaceae	Pan-oot	Rhizome	4.2
3. <i>Allium cepa</i> Linn.	Liliaceae	Kyetthunni	Rhizome	4.2
4. <i>Colocasia antiquorum</i> Schott.	Araceae	Peinmahawyar	Rhizome	4.2
5. <i>Gloriosa superba</i> Linn.	Liliaceae	Hsinmeedauk	Rhizome	4.2
6. <i>Allium sativum</i> Linn.	Liliaceae	Kyetthunbyu	Rhizome	4.2
7. <i>Polianthus tuberosa</i> Linn.	Amaryllidaceae	Hninpan	Rhizome	4.2
8. <i>Dioscorea</i> sp.	Dioscoreaceae	Kyway	Rhizome	4.2
9. <i>Cyperus</i> sp.	Cyperaceae	Myatmonhnyin	Rhizome	4.2
10. <i>Curcuma longa</i> Linn.	Zingiberaceae	Nanwin	Rhizome	4.2
11. Unidentified		Tawkhatta	Rhizome	4.2
12. <i>Amorphophallus campanulatus</i> Blume	Araceae	Wa	Rhizome	4.2
13. <i>Crinum asiaticum</i> Linn.	Amaryllidaceae	Koyankyee	Rhizome	4.2
14. <i>Antidesma</i> sp.	Euphorbiaceae	Kinpalin	Root	4.2
15. <i>Datura fastuosa</i> Linn.	Solanaceae	Padaingnet	Root	4.2
16. Unidentified		Gyatnet	Root	4.2
17. <i>Acacia farnesiana</i> Willd.	Mimosaceae	Nanlonkyaing	Root	4.2
18. <i>Embllica officinale</i> Gaertn.	Euphorbiaceae	Zibyu	Root	4.2
19. Unidentified	Compositae	Pannyo	Leaf	4.2
20. <i>Pogostemon patchouli</i> Pell.	Labiatae	Thanatpyitsee	Leaf	4.2
21. Unidentified		Yaungbyuhsay	Leaf	4.2
22. Calcium oxide		Maphaukhton		4.2
23. <i>Antiaris toxicaria</i> (Pers) Lesch.	Moraceae	Hmyaseik	Latex	4.2
24. <i>Moschus moschiferus</i> Linn.	Cervidae	Kadoe	Dried secretion from the preputial follicles of the musk deer	4.2

San-Khin [9], Hundley and Chit-Ko-Ko [10]

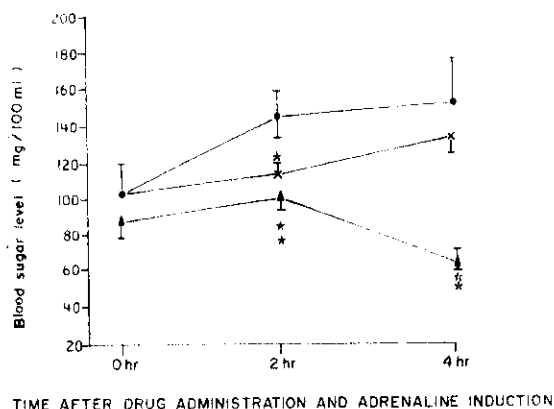


Fig. 2. Time course of the IMF-32 watery

extract and standard drug on adrenaline-induced hyperglycemia rat model.

Each point represents the mean of observations and vertical bar indicates the standard error of the mean. The groups tested were ●-●, placebo; x-x, IMF-32 watery extract and ▲-▲, tolbutamide respectively.

*denotes $p < 0.05$

**denotes $p < 0.025$; $p < 0.005$

that in the control animal, the dose of adrenaline caused increase in blood sugar to the extent of 57 and 60 mg% respectively. These hyperglycemic response of adrenaline were found to be quite significant ($p < 0.005$) in both the animals tested. Statistical analysis of the data revealed that tolbutamide caused a remarkable significant inhibition ($p < 0.025$) of the hyperglycemic response to the induction of adrenaline. Moreover, the inhibition of the hyperglycemic response of TMF-32 watery extract was comparable with that of tolbutamide. With the extract, the peak of action is at 2 hr ($p < 0.05$) in both the animals tested.

Normally, only crude powders are being used for diabetic patients with the dosage of 40 mg/kg. The dose tested in animals models were forty times that of the human dose which was 1.6 g/kg. The maximum feasible dose tested, showed only a slight lowering of blood sugar level when compared with that of the control group. Repeated administration of the above dosage was impossible due to a large amount of suspension. In order to obtain a significant hypoglycaemic effect, the four folds of the 1.6 g/kg was extracted with water. The water soluble extract obtained from 6.4 g/kg of powder was administered orally. By administering the water soluble extract which was 1.4 fold greater than that of the powder showed a significant lowering of blood sugar level in the animals tested. It showed that the watery extract was more potent than that of the TMF powder.

Hyperglycaemia due to adrenaline is known to be effected partly through release of adreno-corticoids as stated by Gupta *et al.* [6], and partly through stimulation of the adreno-hypoglyseal axis as stated by

Voght [7]. Thus, the action of TMF-32 may probably be effective through either direct glycogenolysis or indirectly through action on the adrenocortico-hypoglyseal axis by inhibiting the gluconeogenesis. The exact mechanism of action still need to be further investigated. Tolbutamide has onset of action at 3 to 5 hours and duration of action 6-12 hours as stated by Karam [8]. Therefore, with regard to efficacy and duration of action the TMF-32 extract is not superior to tolbutamide.

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Decontamination of vegetables by washing with natural water

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In Myanmar, raw vegetables are usually consumed after washing with water. The purpose of this study is to determine the degree of decontamination of these vegetables after washing with different types of natural water. The samples of the vegetables from a local market in Yangon were collected and faecal coliforms (FC) were isolated from them using standard methods. FC counts ranged from 2.1×10^2 to 1.1×10^5 /gm of vegetables. The FC isolation rate was reduced by 1 log only after they were washed once with uncontaminated tap water (FCC = 0 MPN/100 ml). But, FC isolation rate of the coriander increased after washing with the contaminated pond and river water. However, a significant reduction could be achieved after washing 3 times with uncontaminated tap water without washing hands and utensils with soap and respective water. But after hands and utensils were washed properly, the isolation rate from vegetables was significantly reduced after washing twice. The use of contaminated water was not effective in decontaminating them.

INTRODUCTION

People in Myanmar usually eat certain raw vegetables (Toe-sa-ya) with salted fish paste (ngapi). These vegetables are often contaminated from the water used for watering/irrigation, from manure or from their handlers. The usual method of rinsing them in natural water before consumption is probably not effective in decontaminating them. The present report is on the study carried out to determine the degree of decontamination of these vegetables after washing with different types of natural water.

MATERIALS AND METHODS

Vegetable samples were collected during March 1989 to February 1990 from a small market in Hlaing

Township in Yangon. There were shops selling vegetables such as coriander, green chilli, salad, leaves of lemon, leek, morning glory, cabbage, legume, mimosa and putinand. The samples were bought randomly in sterile plastic bags from each of the above vendors. A similar collection was repeated on the same day each month from the same vendors. They were collected in the morning and transported to the laboratory for bacteriological processing on the same day.

Twenty five grams of vegetable sample was weighed and 10% suspension was made by using 225 ml of $\frac{1}{2}$ strength Ringer's solution. Another 25 gms of vegetable was washed only once with a measured volume of tap water to obtain a weight/volume ratio of 1:20 and then 10% suspension was made. The faecal coliform

counts (FCC) of the suspensions were determined by International Commission on Microbiological Specifications for Foods method [1].

FCC was done on 10% suspension of coriander after washing only once with different types of natural water (amount of water in washing = w/v - 1:20). It was also done on 10% suspensions of coriander and morning glory after washing twice and 3 times with natural water (amount of water in washing = w/v - 1:20). In all the above experiments, hands and using bowl were not washed with soap and respective water.

Hands were washed with soap and respective water using the modified method [2]. The bowl which was used for washing vegetables was also washed properly with soap and respective water. 25 gms of coriander and morning glory were washed twice and 3 times with respective water in this bowl with thoroughly washed hands, then 10% suspensions were made and FCC was determined (amount of water in washing = w/v - 1:20).

The statistical significance test of differences in FC counts by washing were done using the median test [3] which shows if 2 independent groups differ in central tendencies. This non-parametric test was used because of marked deviations in the FC count data.

RESULTS

All the raw vegetables tested were contaminated and their median FCC ranged from 2.1×10^2 to 1.1×10^5 /gm. FCC decreased only 1 log after they were washed (once) with the tap water having a median FCC of 0 MPN (Most Probably Number)/100 ml (Table 1). The isolation of FCC

Table 1. Isolation of faecal coliforms (FC) (Most probable number: MPN/gm) from vegetables before and after washing (only once) with tap water, Yangon, 1989-90

Type of vegetables	Median FC count	
	before washing	after washing
Coriander	1.1×10^5	2.4×10^4
Green chilli	1.6×10^5	0.7×10^4
Salad	1.1×10^4	2.4×10^3
Leaves of lemon	0.6×10^4	1.1×10^3
Leek	1.7×10^3	0.9×10^3
Morning glory	1.3×10^3	3.5×10^2
Cabbage	0.8×10^3	1.2×10^2
Legume	0.7×10^3	1.3×10^2
Mimosa	2.4×10^2	1.2×10^2
Putinand	2.1×10^2	43

¹Median of 6 days results of each vegetable
FCC of tap water = 0 MPN/100 ml

Table 2. Isolation of faecal coliforms (FC) (most probable number: MPN/gm) from vegetable-coriander before and after washing (only once) with natural water, Yangon, 1989-90

Type of natural water	¹ Median FC count		
	Water (MPN/100 ml)	Coriander (MPN/gm)	
		before washing	after washing
Tap	0	1.1×10^3	0.9×10^2
Well	0.2×10^2	0.2×10^4	1.5×10^3
Pond	2.4×10^3	0.4×10^3	1.1×10^3
River	2.4×10^3	0.9×10^3	2.4×10^3

¹Median of 6 days results

from coriander before and after washing (once) with natural water is shown in Table 2. FC isolation rate increased when it was washed with pond and river water. But these difference in FC counts from

Table 3. Isolation of faecal coliforms (FC) (Most probable number: MPN/gm) from vegetables coriander and morning glory before and after washing with natural water, Yangon, 1989-90

Type of natural water	¹ Median FC count						
	Water (MPN/100 ml)	Coriander (MPN/gm)			Morning glory (MPN/gm)		
		Raw	2W3	3WHU	Raw	2W3	3WHU
Tap	0	1.2×10^3	23	0	0.4×10^3	0	0
Well	15	2.4×10^3	93	43	0.9×10^2	0.5×10^3	9
Pond	2.4×10^2	1.9×10^3	93	43	1.5×10^2	1.5×10^3	43
River	2.4×10^3	3.1×10^3	1.1×10^3	2.4×10^2	0.6×10^3	0.4×10^3	0.2×10^3

1 Median of 6 days results

2 Washing 3 times with respective water only

3. Washing 2 times and washing hands and utensils with soap and respective water

coriander before and after washing were not statistically significant. The isolation of FCC from vegetables-coriander and morning glory before and after washing 3 times with natural water is given in Table 3. The isolation rates were significantly reduced after washing 3 times with tap water (FCC = 0 MPN/100 ml) without washing hands and utensils. But after hands and utensils were washed properly, the isolation rate from vegetables was significantly reduced after washing twice. However, the decontamination of these vegetables with well, pond and river water was not as effective as the tap water. The difference in FC counts from coriander and morning glory before and after washing with natural water were not statistically significant.

DISCUSSION

On the unwashed surfaces of tomatoes, FC counts as high as 5×10^3 /sq cm have been recorded and on washed tomatoes, the FC counts vary between $4-7 \times 10^2$ /sq cm [4]. The results of the present study are similar and

our findings show that there is only 1 log difference between washed (one time) and unwashed vegetables, using the tap water.

A study in Lebanon evaluates the effectiveness of reducing contamination of fruits and vegetables by washing. In general, washing is not effective in reducing the number of samples positive for various bacteria, including *E. coli*. For instance, 50% of washed lettuce samples but only 29% of unwashed samples were positive for *E. coli*. Vegetables were washed using tap water, but the tap water had not been tested for the presence of *E. coli* [5]. We also found that FCC was not reduced when the coriander was washed with contaminated well, pond and river water.

Contamination of hands with pathogenic agents of diarrhoea have been documented [6]. However, thorough hand washing with soap and water is found to be effective in decontaminating the hands [7]. Efforts to promote hand washing and cleaning of utensils may result in reduced contamination [8].

We found that raw vegetables can only be decontaminated after washing 3 times with the tap water. It is of the utmost importance that such vegetables be well washed before consumption. We high-light the importance of washing hands and utensils also in decontamination of raw vegetables and also the significance of the water quality and quantity for improving the domestic hygiene in developing countries.

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Purification of enterotoxigenic Escherichia coli
heat labile toxin (Part 1)

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Purification of Escherichia coli heat labile toxin (LT) was done from 9 litres of culture. The cells were suspended in 0.01M Tris (hydroxymethyl) amino-methane (Tris)-hydrochloride buffer (pH 8.6) containing 0.9 percent sodium chloride. The suspension was sonicated. The supernatant was then treated with ammonium sulphate and dissolved in TEAN buffer and dialysed. This crude LT was then passed through ASM BioGel and was collected by D-galactose and concentrated to about 3 ml. It was then passed through Sephacryl S-200 column and again concentrated by Amicon PM 10 membrane and used as purified LT. Separation of subunits were carried out by using 6M urea solution in 0.1M propionic acid (pH 4.0). Every steps of fractions were checked by Ouchterlony double gel diffusion test using anti cholera sera and by CHO cell assay. Protein assay was done and specificity of the protein was also checked by SDS polyacrylamide slab gel electrophoresis.

INTRODUCTION

The two virulence factors namely (a) the ability to colonize the small intestine and (b) the production of enterotoxin give the pathogenicity of Escherichia coli to cause acute diarrhoea. It was shown that the heat labile enterotoxin known as LT produced by certain strains of E. coli is a protein remarkably like cholera toxin [1,2,3]. It contains several B subunits of 11,780 molecular weight [4] and A subunit, estimated as 25,500 [5] to 30,000 [6] molecular weight. Its subunit number and arrangement was shown by Gill *et al.*, 1981 [7]. The hybrid toxins between subunits of E. coli heat labile enterotoxin and those of cholera toxin (CT) was performed by Takeda [8] and demonstrated that the hybrid toxin show a similar toxicity to that of the parent toxins from

which the A subunits are derived. The immunological and biological similarities of LT and CT were also well established [9-19].

MATERIALS AND METHODS

Bacterial strain and culture of cells

Escherichia coli W165-1/82 from acute diarrhoea case from hospital, Yangon was cultured in 9 litres of Casamino Yeast Extract (CAYE) medium with vigorous shaking (Takasaki shaker, 280 rpm/min.) at 37°C for overnight. CAYE media contained 2 percent Casamino acids (Difco Laboratories); 0.6 percent yeast extract (Difco); 0.25 percent sodium chloride; 0.871 percent dipotassium hydrogen phosphate; 0.25 percent glucose and 0.1 percent (v/v) trace

salt solution (5 percent MgSO_4 , 0.5 percent MnCl_2 , 0.5 percent FeCl_3 and 0.001 percent H_2SO_4). The medium was supplemented with 90 μg of lincomycin hydrochloride per ml of media (UpJohn Ltd., Tokyo) to enhance the synthesis of LT [16].

Preparation of crude E. coli heat labile toxin (LT)

Crude LT was prepared from whole cell lysate as it is the richest source of LT as noted by Clements & Finkelstein, 1978 [9]. Approximately 9 litres of bacterial culture was centrifuged at 10,000 rpm for 20 minutes at 4°C and pelleted cells were suspended in approximately 300 ml of 0.01M Tris (hydroxymethyl) aminomethane (Tris)-hydrochloride buffer (pH 8.6) containing 0.9 percent NaCl. The suspension was sonicated in an ultrasonic disrupter sonicator (Model UK-200P Tomy) for 5 minutes on ice. The sonicated suspension was spun at 15,000 rpm for 30 minutes and the supernate was then spun for 20,000 rpm for 5 hours. The supernatant was then collected and solid ammonium sulphate was added to give 65 percent saturation. The precipitate was collected by spinning at 10,000 rpm for 30 minutes and suspended in about 200 ml of 0.05M Tris-HCl buffer pH 7.4, containing 1 mM ethylenediaminetetraacetic acid 3 mM sodium azide and 0.2M sodium chloride (TEAN buffer). It was then dialyzed twice against 2 litres of TEAN buffer for about 4 hours and used as crude LT preparation.

Purification of E. coli heat labile toxin (LT)

LT was purified partially by passing 200 ml of crude LT to a BioGel A 5M column (90 x 200 mm) equilibrated with TEAN buffer. The column was washed with 400 ml of TEAN buffer and LT was then eluted with 400 ml of 0.3M D-galactose solution in TEAN

buffer [1]. Fractions containing LT was assayed by Ouchterlony double gel diffusion test with anti-purified cholera toxin antisera (prepared at DMR by immunization rabbits with pure cholera choletox toxin purchased from the Chemo-Sero-Therapeutic Research Institute); were collected and concentrated to about 3 ml by Amicon PM 10 membrane filtration (Amicon Corp., Lexington, Mass.). The preparation was then applied to a Sephacryl S 200 column (90 x 200 mm) equilibrated with TEAN buffer. The column was eluted with the same buffer and the fractions containing LT were collected, concentrated by Amicon PM 10 membrane filtration and used as purified LT.

Separation of A and B subunits

15 mg of LT toxin in 5 ml of TEAN was dialysed against 200 ml of 6M urea solution in 0.1M propionic acid pH 4.0 for about 16 hr and then against 200 ml of the same solution for 6 hr. Spectra/pore membrane tubing, which cuts off molecules with molecular weights of less than 1,000 was used for dialysis. The dialysed preparation was applied to a Sephadex G-75 column (100 x 2.5 cm) equilibrated with 6M urea solution in 1.0M propionic acid (pH 4.0), and material was eluted with the same solution [20].

Specificity of enterotoxigenic Escherichia coli (ETEC) toxin

The specificity of the proteins was checked by Ouchterlony double gel diffusion test [21]. The sensitivity was checked by Chinese Hamster ovary cell assay as according to the method of Guerrant *et al.*, 1974 [22] and modified by Honda *et al.*, 1976 [23]. The protein amount was assayed by the method of Lowry *et al.*, 1951 [24]. The specificity of the proteins was then checked by gradient SDS polyacrylamide slab gel

electrophoresis as the method of Laemmli, 1970 [25].

Preparation of anticholera sera

Anti-CT was prepared by the method described by Hamashima, 1976 [26]. After obtaining anti CT, the fractions of LT were checked by Ouchterlony double gel diffusion test. The titre of the antisera was checked and determined as described by Honda *et al.*, 1981 [27]. The sensitivity and specificity of the antisera were checked by Ouchterlony double gel diffusion test [25] and immunoelectrophoresis by Hamashima 1976 [26].

Immunoaffinity column chromatography

It was carried out [16] as 1 gram amount of cyanogen bromide activated Sepharose 4 B (Pharmacia) was coupled with 7 mg of the purified antigen. Antisera was applied to a column and the immunoglobulin bound to the column was then eluted with 0.2M glycine-hydrochloride buffer (pH 2.7) containing 0.5M sodium chloride. After recovery of specific antisera, pH was readjusted to about (7.20) as soon as possible. Neutralizing test using antisera in CHO cells with specific antigens was also done to know the specificity and sensitivity of the antisera and antigen.

RESULTS

Figure 1 shows the production of purified LT after passing through Biogel A5M column. The small fraction so obtained was collected and checked for its specificity and sensitivity by Ouchterlony and cell assay. It was then passed through Sephacryl S 200 column.

Figure 2 shows the precipitin line obtained by testing the fractions with anti cholera toxin. Three

preparation of pure LT were introduced and compared with CT and found that they shared a common antigen but that each also has a unique antigen. CT and LT were antigenically related but not identical. The patterns might indicate the presence of multispecific antigen in one of the reactants compared.

Figure 3 shows the SDS gradient gel electrophoresis in comparison with cholera toxin. It was found that the molecular weight of subunits A and B of LT Possess 31 k and 11 k respectively.

DISCUSSION

The heat labile enterotoxin (LT) of *E. coli*, like cholera toxin (CT) of *V. cholera*, stimulates the production of cAMP in intestinal mucosal cells by ADP-ribosylating the 45-kDA G α component of the stimulatory regulatory subunit of the adenylate cyclase regulation system [28,29]. This results in a loss of water and electrolyte from the cells [30,31].

LT resembles CT not only functionally, but structurally and immunologically as well [6,7,9,32,33]. Both toxins contain dissimilar subunit, A and B, probably present in the holotoxin in a ratio of 1A to 5B [3,33]. The B subunits are responsible for the binding of the toxin to its target cells via Gm₁ ganglioside [29] while the A subunit bears the enzymatic (toxic) activity.

In spite of the high B and A subunit ratio, both CT and LT are translated from single polycistronic messengers coding for one A subunit, followed downstream by one B subunit [4,34-36]. Nucleotide sequences of their respective genes predict that both LT and in CT, the A and the B subunits are synthesized as precur-

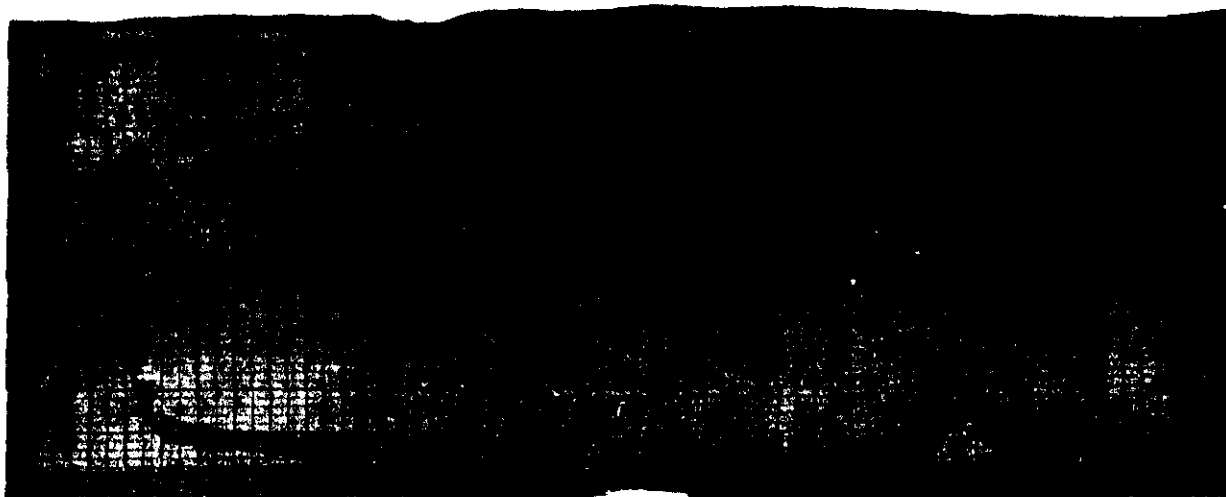


Fig. 1. Purification of *E. coli* heat labile enterotoxin after passing through the Biogel A5M

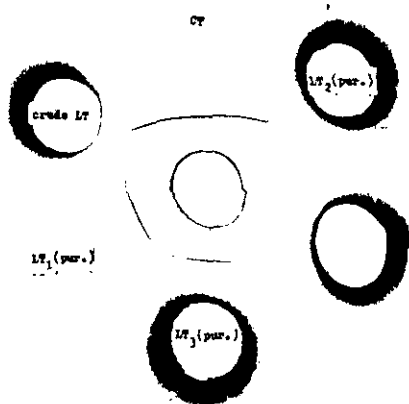


Fig. 2. Ouchterlony double gel diffusion showing the precipitin lines by testing LT antigen, CT antigen with anti-cholera toxin

sor proteins with signal sequences of 18 aminoacids for the A subunit and 21 amino acids for the B subunits [4,35,36].

Though CT and LT bear extensive similarities in protein chemistry, they differ considerably in their localization. While CT is released from *V. cholerae* cells and occurs in the surrounding medium [37,38], LT remains cell associated in *E. coli*. LT occurs free in the periplasmic

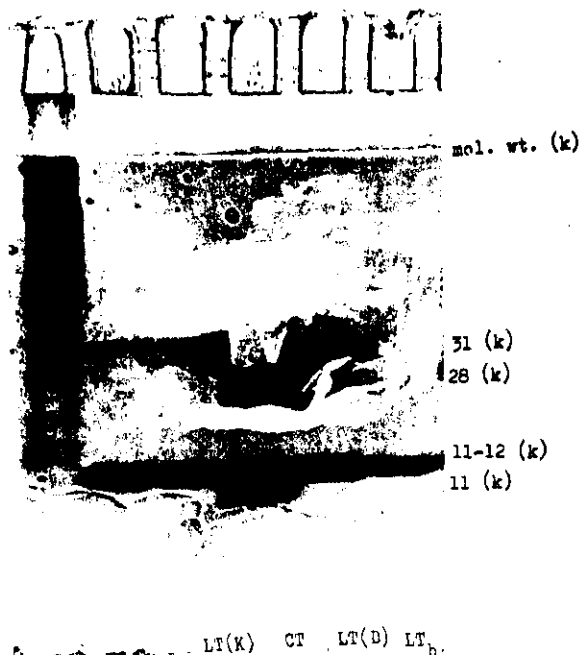


Fig. 3. SDS gradient gel electrophoresis showing a comparison with cholera toxin and LT toxin

space not bound to the cytoplasmic or outer membrane [39,40].

Hofstra & Witholt [41] also studied the kinetics of association of subunits into periplasmic holotoxin

and explained that LT subunits are translocated and processed in a ratio of about 2A to 5B. Since free LT A is either degraded slowly on bound to newly synthesized LTB oligomers, the net result is a steady state of 1.4 to 1.7 A subunits to 5B subunits in the periplasm and 60 percent of LTA is bound by LTB to form periplasmic holotoxin with a subunit ratio of about 1A to 5B. The remaining 40 percent of periplasmic LTA occurs free.

Our main aim for the purification of LT of E. coli is to raise the antibody for use in the identification of enterotoxin (LT) producing E. coli.

It was also reported by other workers that when used with porcine LT antiserum or CT antisera the results did not give a definite precipitation lines probably because the precipitation antigen in the human LT was too weak [16,19].

It was also observed that by SDS electrophoresis, the subunits A of CT and LT shows different molecular weights as was found by other investigators. It was also shown that CT and LT are immunologically related but not identical and showed that they share a common antigen determinant(s) and in addition, that each enterotoxin possesses its own antigen determinant(s). LT is antigenically similar to cholera toxin in that antibodies prepared against either toxin cross neutralize to both toxic activities. LT must therefore possess a region that is antigenically similar to at least a portion of cholera toxin subunit B. As well as having characteristics of cholera toxin subunit B, E. coli LT appears to possess properties very similar to those of fragment A of cholera toxin. The similarities between LT and CT are

obviously great and investigators [10-18] suggest at least a common ancestors for those two toxins. Indeed LT may differ functionally from CT only in its lack of a fragment A₂ like moiety.

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Preparation of enterotoxigenic Escherichia coli
(heat labile) antisera and its use in
BIKEN assay (Part 2)

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The purified Escherichia coli (LT) toxin was immunized in rabbits. The specificity of the antisera was demonstrated by Ouchterlony double-gel diffusion tests and by neutralization of the activities of CT and LT to cause morphological changes in Chinese Hamster Ovary cells assays. The optimum titre of the antisera was checked and BIKEN assay was used to check the sensitivity and specificity of the antisera. A total of 965 isolates were used to checked for the identification of LT by BIKEN assay and found that 27 isolates produced LT.

INTRODUCTION

Gyles and Barnum [1] first suggested that cholera toxin (CT) and heat labile toxin (LT) of enterotoxigenic Escherichia coli were immunologically related by demonstrating of porcine E. coli LT activity of anti CT antiserum. Clements and Finkelstein [2] clearly showed that LT is immunologically related to subunits A and B of CT neutralization of activities and by double-gel diffusion test. Honda *et al.* [3] developed a BIKEN test (modified ELEK) for the detection of Enterotoxigenic E. coli (ETEC) producing heat labile toxin in 1981. Honda *et al.*, 1981 [4] also showed the use of anti LT which showed more specific reactions toward LT producing E. coli than anti cholera toxin. The specific antiserum is required as there are also immunological non-identity of heat labile enterotoxin such as human and porcine Enterotoxigenic E. coli.

MATERIALS AND METHODS

Preparation of anti LT antisera

It was done as described by Takeda *et al.*, 1981 [5] 25 ug of pure LT was emulsified with an equal volume of Freund complete adjuvant (Difco) and the emulsion was inoculated at the back of the rabbits. Two booster injections were given on days 25 and 50 with 25 ug of toxin emulsified with Freund complete and incomplete adjuvants (Difco) respectively. Antisera was obtained on day 60.

Immunoaffinity column chromatography

It was done as described by Honda *et al.*, 1976 [6].

Determination the optimum titre of antisera

It was done by Ouchterlony double gel diffusion test. 2 ug of the pure LT was placed in the centre

well and different dilutions of the antisera was placed in the peripheral wells and kept in a humidified incubator at room temperature for 16-24 hrs.

BIKEN assay

It was done as according to the method of Honda *et al.*, 1981 [3]. Four strains of *E. coli* to be tested were inoculated onto a single plate of BIKEN AGAR No. 2. The plate was incubated at 37°C for 48 hrs, paper disks (Whatman AA) containing polymyxin B were placed on each colony, and the plate was incubated for an additional 5 hr. The antisera against purified human LT was put into a well cut close to each colony (about 5 mm in distance, and the plate was further incubated at 37°C for 24 hr. Precipitin lines between anti LT serum and bacterial colonies were usually examined without magnification. In some instances, the plates were washed extensively against a solution containing 0.4 percent NaCl and 0.4 percent Na₂B₄O₇; dried, stained and destained as described by Takeda *et al.*, 1981 [5].

The maximal dilution of antisera that gave a precipitin line against 2 ug of purified LT in the Ouchterlony gel diffusion was checked and used in the BIKEN assays [7].

Identification of heat labile enterotoxin

For standardization of the antisera 124 standard strains of *E. coli* were tested in BIKEN assay in parallel with the CHO cell assay. Moreover, 965 isolates of *E. coli* isolated from Intakaw (cool dry) were tested for the production of heat labile enterotoxin by BIKEN assay and CHO cell simultaneously [8].

RESULTS

Figure 1 represents the determination of the optimum titre of antisera. 2 ug of the antigen was placed in the centre well and different dilution of antisera was placed in the peripheral wells. It was found that the optimum titre of antisera was found to be 1/8. The antisera so obtained was also tested in CHO cell assay for neutralization and found that it neutralized well up to the titre of 1:512, using both cholera toxin and *E. coli* (LT) toxin.



Fig. 1. Determination of the optimum titre of anti LT antisera

Figure 2 shows the specificity of anti LT after raising in rabbits by Ouchterlony double gel diffusion tests. It was shown as similar results with others that CT and LT are antigenically related but not identical. They share a common antigen determinant(s) that is not present as one of the antigen and shows that each enterotoxin possesses its own antigen determinant(s). Figure 3 shows the precipitin line when used with anti LT_b and Figure 4 illustrates the specificity of antisera by immunoelectrophoresis.

Figure 5 represents the BIKEN method for the identification of *E. coli* heat labile toxin. The *E. coli* strain was placed in 0, 1 represents

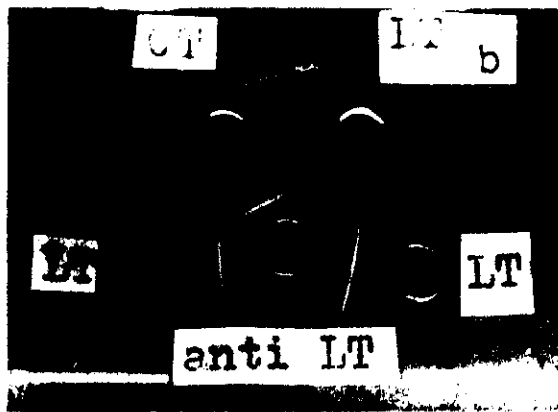


Fig. 2. Specificity of anti LT by Ouchterlony double gel diffusion test

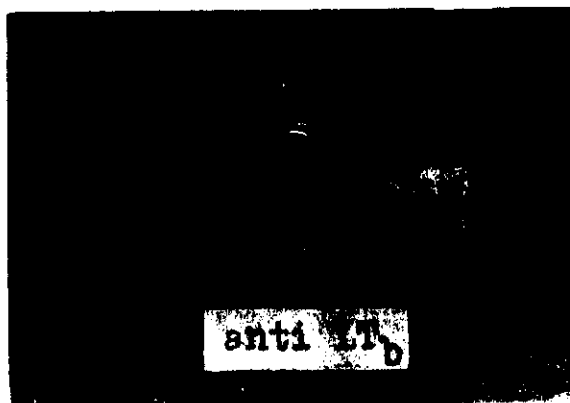


Fig. 3. Specificity of anti LT_b Ouchterlony double gel diffusion test

a control antisera and 2 represents the anti LT antisera prepared at DMR. It was found that anti LT showed a good response in BIKEN assay.

Table 1 shows the use of BIKEN method in detecting E. coli heat labile toxin in children with diarrhoea. A total of 965 isolates of E. coli isolated from children with diarrhoea were tested for heat labile toxin by CHO cell assay and BIKEN assay. It was found that 27 isolates of E. coli showed LT both by BIKEN and by CHO cell assay.

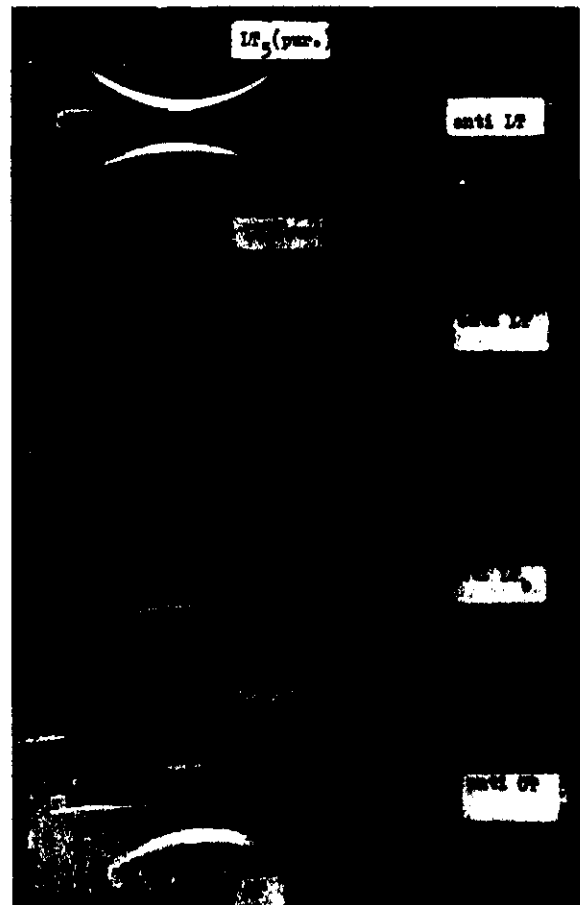


Fig. 4. Specificity of antisera by immunoelectrophoresis



Fig. 5. BIKEN method for the identification of E. coli heat labile toxin

Table 1. Comparison of BIKEN and CHO test for detecting E. coli toxins (heat labile)

<u>E. coli</u> Type	No. of isolate	BIKEN	CHO
LT	6	6	6
ST	31	0	0
STLT	21	21	21
Nontoxigenic	907	0	0
Total	965	27	27

DISCUSSION

The heat labile enterotoxin (LT) of E. coli acts by stimulating adenylate cyclase activity and so, superficially at least resembles to cholera toxin. LT is antigenically similar to cholera toxin in that antibodies prepared against either toxin cross neutralize to both toxic activities. LT must therefore possess a region that is antigenically similar to at least a portion of cholera toxin subunit B. As well as having characteristics of cholera toxin subunit B, E. coli LT appears to possess properties very similar to those of fragment A1 of cholera toxin. The immunological and biological similarities between cholera enterotoxin (CT) and E. coli heat labile enterotoxin (LT) have been demonstrated by many workers. The similarities between LT and CT are obviously great and they suggested at least a common ancestor for these two toxins. Indeed LT may differ functionally from CT only in its lack of a fragment A₂-like moiety.

Without question diarrhoeal diseases constitute one of the greatest causes of morbidity and death on a global scale. Diarrhoeal diseases, which are often caused by infectious microorganisms or their toxins, constitute the greatest causes of mortality and one of the greatest

causes of morbidity on a global scale. It was reported by Walsch & Warren in 1979 [9] that in, Africa, Asia and Latin America 3 to 5 billion got illness and 5 to 10 million deaths occurred. Snyder and Merson in 1982 [10] reported that among 338 million of children under 5 years of age 0.7 to 1 billion got illnesses and 4.6 million of deaths occurred. In 1983 Guerrant et al. [11] also demonstrated that in NE Brazil children got 6 episodes per child per year and the 7.7 to 13 percent of deaths occurred. Depending on age and living conditions, average attack rates of diarrhoeal illness exceed to one to six episodes per person per year and diarrhoeal illness account for the greatest portion of the staggering childhood mortality that exceeds 20 percent in the poorest areas.

In Myanmar, it was reported the importance of ETEC as an aetiological agent in acute diarrhoea in children by Tin Aye et al. [12,13]. It was also reported the reliability of the use of BIKEN ASSAY in detecting heat labile enterotoxin of Escherichia coli by Sutton et al., 1985 [14] and it can be used as a diagnostic tool in laboratories without assay sophisticated equipments. Honda et al. [7] reported the usefulness of the BIKEN test for detection of LT-producing E. coli in a large scale field study. They showed in sharper and clearer precipitin line than did anti CT when anti LT was used. This was probably because in addition to an antigen determinant(s) common to both LT and CT, LT and CT each has a specific antigen determinant. BIKEN assay has now been used extensively by many investigators and found to be convenient and reliable [14-17]. Takeda et al. [18] tested 2229 isolates of E. coli isolated from diarrhoeal patients

and the results showed 99.0 percent coincidence with those obtained by CHO cell assay. In our study also there is a good relationship between the results of CHO cell assay and BIKEN assay.

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Disinfectants efficacy on bacteria with the contaminants

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The study was carried out to determine the effect of contaminants on the efficacy of routinely used disinfectants on bacteria and call on attention of hospital staff and medical care personnel for correct usage of disinfectants. Disinfectant efficacy of Hibitane, Osban, Ethanol, Isodine and Tego 51 on organisms such as *P. aeruginosa*, *E. coli*, *S. aureus* and *B. subtilis* contaminated with organic substances such as human blood, egg white and animal oil were investigated. The disinfectant efficacy was also determined on clinical specimens. 70% Ethanol was totally ineffective on *B. subtilis*. Osban and Ethanol did not effect on *P. aeruginosa*, and *B. subtilis* when they were contaminated with the organic substances. Tego 51 was weak in the efficacy on sputum and urine specimens. Hibitane and Isodine were effective on tested organisms even when contaminated with organic substances and also on the clinical specimens.

INTRODUCTION

Disinfectants are widely used not only for medical purposes but also for domestic usage. Many workers have investigated efficacy of disinfectants on pathogens which are mostly normal inhabitants in environment and in human but seriously concerning with nosocomial infections in immune compromised host. Contaminants such as urine, stool, pus etc. reduce the disinfectants efficacy on such pathogens [2]. There has been little information about the effect of organic contaminants on the bactericidal activity of disinfectants. Thus this study has been carried out to make clear the effect of contaminants on the efficacy of routinely used disinfectants and call on attention to hospital staff and medical care personnel for correct usage of disinfectants.

MATERIALS AND METHODS

The final concentration of disinfectants namely Habitane, Osban, Ethanol, Isodine and Tego 51 were used at 0.5%, 0.1%, 70%, 50% and 0.2% respectively. *P. aeruginosa*, *E. coli*, *S. aureus* and *B. subtilis* were used to test the disinfectant efficacy on bacteria. The animal oil, egg white and blood (sterile human blood) were used as contaminants and then urine, sputum, stool and pus were used as hospital specimens. The hospital specimens were collected from Tsukuba University Hospital. *P. aeruginosa*, *E. coli*, *S. aureus*, *B. subtilis* were inoculated in brain heart infusion (BHI) media and incubated at 37°C for overnight. They were diluted with BHI media to get the working concentration of 10^7 , 10^8 , 10^9 colony forming unit/ml (CFU/ml) for each organism by

measuring absorbance at 540 nm. 1 ml of each organism suspension was mixed well by vortexing with 1 ml of working concentration of each disinfectant. After 10, 30, 60 and 180 secs, 100 ul of the mixture was spreaded on nutritient agar (NA) and then incubated at 37°C for overnight. Bactericidal effect was calculated from the colony number grown on NA plates after overnight incubation.

The final concentration of human blood, egg white and animal oil as contaminants were used as 10%, 10% and 50%, respectively. A half ml of 10^9 CFU/ml of each tested organism in BHI media broth was mixed well by vortexing with a half ml of each contaminant. Then 1 ml of final concentration of each disinfectant was added to the above mixture and mixed well by vortexing. After 10, 30 and 180 secs of mixing, 100 ul of the mixture was spreaded on NA plates and incubated at 37°C for overnight. Bactericidal effect was determined by the number of colonies on NA plates after overnight incubation.

Patient's sputum, urine, stool and pus were examined for the efficacy of disinfectants. Total bacterial count of these specimens were measured on NA by CFU assay. 1 ml of 10% of each clinical specimen was mixed well by vortexing with 1 ml of final concentration of each disinfectant. After 10 secs of mixing well 100 ul of the mixture was spreaded on NA plates and incubated at 37°C for overnight. Then bactericidal effect was measured as mentioned above. If there was any growth of bacterial on these plates, those were identified biochemically by standard procedures [1].

RESULTS

The results of efficacy of disinfectants on the tested organisms were

shown in Table 1. At the tested concentrations, these disinfectants were effective on tested organisms except that 70% Ethanol which was totally ineffective on B. subtilis on all the concentrations of 10^9 , 10^8 and 10^7 CFU/ml.

The efficacy of disinfectants with the contaminant was shown in Table 2. Osban did not effect on P. aeruginosa when it was mixed with these organic contaminants. Ethanol was not effective on B. subtilis when it was contaminated with the organic substances. Osban and Tego 51 did not effect on E. coli and on P. aeruginosa respectively when they were contaminated with oil.

The efficacy of disinfectants on the clinical specimens were shown in Table 3. Tego 51 was weak in the efficacy on sputum and urine specimens when the total bacterial count in the specimens reached to 10^8 and 10^9 CFU/ml. The contaminated bacteria were identified to be P. aeruginosa and E. coli.

Hibitane 0.5% and Isodine 50% were effective on tested organisms even when contaminated with organic substance and also on the clinical specimens.

DISCUSSION

Hibitane is biguanides and it is strongly positive charged disinfectant. It inactivates the membrane proteins of the bacterial cells. It is not noxious to skin and is commonly used one. Its weak point is that it is easily contaminated by pathogens. It has a wide spectrum of activity against gram negative and positive bacteria but it is not sporicidal and mycobactericidal. Its potency is reduced in the presence of serum, blood, pus and other organic matter. Because of its

Table 1. Bactericidal effect of disinfectant on common bacteria at various concentration of the organism

Disinfectant	Organism	Concentration of Organism/Duration of Treatment (sec)											
		10 ⁹ CFU/ml				10 ⁸ CFU/ml				10 ⁷ CFU/ml			
		10	30	60	180	10	30	60	180	10	30	60	180
Hibitane (0.5%)	<u>P. aeruginosa</u>	-	-	-	-	-	-	-	-	-	-	-	-
	<u>E. coli</u>	-	-	-	-	-	-	-	-	-	-	-	-
	<u>S. aureus</u>	-	-	-	-	-	-	-	-	-	-	-	-
	<u>B. subtilis</u>	-	-	-	-	-	-	-	-	-	-	-	-
Osban (0.1%)	<u>P. aeruginosa</u>	-	-	-	-	-	-	-	-	-	-	-	-
	<u>E. coli</u>	-	-	-	-	-	-	-	-	-	-	-	-
	<u>S. aureus</u>	-	-	-	-	-	-	-	-	-	-	-	-
	<u>B. subtilis</u>	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol (70%)	<u>P. aeruginosa</u>	-	-	-	-	-	-	-	-	-	-	-	-
	<u>E. coli</u>	-	-	-	-	-	-	-	-	-	-	-	-
	<u>S. aureus</u>	-	-	-	-	-	-	-	-	-	-	-	-
	<u>B. subtilis</u>	+	+	+	+	+	+	+	+	+	+	+	+
Isodine (50%)	<u>P. aeruginosa</u>	-	-	-	-	-	-	-	-	-	-	-	-
	<u>E. coli</u>	-	-	-	-	-	-	-	-	-	-	-	-
	<u>S. aureus</u>	-	-	-	-	-	-	-	-	-	-	-	-
	<u>B. subtilis</u>	-	-	-	-	-	-	-	-	-	-	-	-
Iego 51 (0.2%)	<u>P. aeruginosa</u>	-	-	-	-	-	-	-	-	-	-	-	-
	<u>E. coli</u>	-	-	-	-	-	-	-	-	-	-	-	-
	<u>S. aureus</u>	-	-	-	-	-	-	-	-	-	-	-	-
	<u>B. subtilis</u>	-	-	-	-	-	-	-	-	-	-	-	-

+ = non bactericidal (organism growth positive after treatment)

- = bactericidal

cationic nature, its activity is reduced in the presence of soaps and other anionic compounds [2]. In our study Hibitane 0.5% was quite effective on tested organisms even contaminated with organic substance and also on the clinical specimens.

Osban (Iodophors) is the positive charged detergent. Because of its lower chemical reactivity it is a weak disinfectant [2]. We found that Osban did not effect on P. aeruginosa when it was mixed with organic contaminants and also on E. coli when it was contaminated with

oil.

70-80% Ethanol gives dehydration of cell membrane. It is extensively employed as a skin disinfectant. For general use, concentrations between 50-70% are recommended as being most effective. One frequently encounters the statement that the presence of water is essential to the antimicrobial action of ethanol, and 70% ethyl alcohol is the most effective bactericidal concentration [3]. In this study, it was quite effective on the tested organisms except that it was totally ineffec-

Table 2. Effect of some contaminants on bactericidal activities of disinfectants

Disinfectant	Contaminant	Bacterial strains tested (10 ⁹ /ml)/Duration of treatment (sec)															
		<i>P. aeruginosa</i>				<i>E. coli</i>				<i>S. aureus</i>				<i>B. subtilis</i>			
		10	30	60	180	10	30	60	180	10	30	60	180	10	30	60	180
Hibitane (0.5%)	Egg White	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Red Cell	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Whole Blood	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Oil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Osban (0.1%)	Egg White	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	Red Cell	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	Whole Blood	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	Oil	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Ethanol (70%)	Egg White	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
	Red Cell	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
	Whole Blood	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
	Oil	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Isodine (50%)	Egg White	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Red Cell	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Whole Blood	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Oil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tego 51 (0.2%)	Egg White	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
	Red Cell	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
	Whole Blood	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	+
	Oil	+	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-

Egg White: 10%, Red Cell: 10%, Whole Blood: 10%, Oil: 50%

+ : non bactericidal (organism growth positive after treatment)

- : bactericidal

tive on *B. subtilis*.

Iodine (Povidone Iodine) is the negatively charged strong detergent. It acts by aggregation and oxidation of proteins. Its action is good in low pH. Povidone Iodine germicide formulated as an antiseptic may contain 10% povidone iodine and 1% available iodine. The term "available iodine" refers to the amount of iodine that is titratable with sodium thiosulphate [2]. In our study Isodine 50% was effective on tested organisms even contaminated with organic substance and also on the clinical specimens.

Tego series of compounds are amphoteric agents which combine the detergent properties of anions with the antimicrobial properties of the cationic compounds. Activity remains virtually constant over a wide pH range and they are inactivated less readily than cationic surfactants by proteins [2]. We found that Tego 51 did not effect on *P. aeruginosa* when it was contaminated in oil. It was also weak in the efficacy on sputum and urine specimens when the total bacterial count reached to 10⁸ and 10⁹ CFU/ml in these specimens.

Table 3. Efficacy of disinfectant on clinical specimens

Specimen	Lab. No.	Contaminated Bacterial Number (CFU/ml)	Disinfectant (final concentration %)					Survived bacteria
			Hibitane(0.5)	Osban(0.1)	Ethanol(70)	Isodine(50)	Iego(0.2)	
Sputum	193	10 ⁷	-	-	-	-	-	
	202	10 ⁵	-	-	-	-	-	
	199	10 ⁶	-	-	-	-	-	
	242	10 ⁷	-	-	-	-	+	<u>Pseudomonas spp</u>
	152	10 ⁸	-	-	-	-	+	<u>S. epidermidis</u>
Urine	232	10 ⁷	-	+	-	-	-	<u>K. oxytoca</u>
	180	10 ⁶	-	-	-	-	-	
	225	10 ⁸	-	+	-	-	+	<u>S. aureus</u>
	220	10 ⁹	-	+	-	-	+	<u>S. aureus</u>
Pus	230	10 ⁹	-	-	-	-	+	<u>S. aureus</u>
	242	10 ⁹	-	-	-	-	+	<u>E. coli</u>
Stool	289	10 ⁹	-	-	-	-	-	
	267	10 ⁹	-	-	-	-	-	

+ = non bactericidal (organism growth positive after treatment)

- = bactericidal

The efficacy of disinfectants on bacteria depends on the hydrophobic properties of the bacteria and the pores of the bacterial membrane where the disinfectant molecules enter into the cell and acts on these cells [2]. So to know the differences of the actions of the above disinfectants on the tested organisms, we need further studies to know the hydrophobicity of the bacterias and also the different pore sizes of them.

From our study we can give some information about the disinfectants to the medical personnels that Hibitane 0.5% and Isodine 50% were quite effective on the tested organisms and also on the clinical specimens. We have found that the organic contaminants and the clinical specimens reduced the efficacy of those disinfectants.

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Enteroadherent Escherichia coli isolated from diarrhoea and dysentery cases in Myanmar

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A total of 419 isolates Escherichia coli, isolated from 173 cases of diarrhoea, 39 cases of dysentery and 21 control cases of different age groups were subcultured in Trypticase Soy Broth containing 1 percent D-mannose. Adherence cell assay was done by using Lab Tek chamber slides seeded with Hep-2 cells. A total of 46 cases (21.7 percent) showed adhesion and noted that 17.5 percent of EPEC, 16.7 percent of ETEC and 8.3 percent of untypable E. coli showed adherence. 28 isolates of them showed diffused adherence pattern and 14 isolates showed localized adherence pattern; 4 isolates also showed aggregative type of adherence.

INTRODUCTION

Adherence has been considered an important factor for the pathogenesis of bacteria on the mucosal surface of the host organism. Escherichia coli strains with the ability to adhere to the human intestinal epithelium have been described in serogroups 026, 0125 and 0119 [1,2,3]. Using tissue culture cells, Cravioto et al. [4] and Mathewson et al. [5] described adherence of E. coli to Hep-2 cells, whereas McNeish et al. [6] observed adherence of the same bacteria to mucosal cells of human fetal small intestine. Scaletsky et al. [7] used HeLa cells to see the distinctive patterns of adherence. Some used Enzyme-Linked Immunosorbant assays for the detection of adhesive factor antigens [8,9]. This paper concerned with the adherence pattern of E. coli isolates of Myanmar diarrhoea and dysentery patients to Hep-2 cells.

MATERIALS AND METHODS

Specimens for bacteriology were inoculated directly onto MacConkey and Salmonella-Shigella agar and into Selenite enrichment broth and incubated overnight at 37°C. Identification of bacteria was followed by the standard methods recommended by the World Health Organization [10] and serotyping was done as according to the methods described in Edwards and Ewing [11].

317 isolates of Escherichia coli isolated from 173 cases of diarrhoea, 72 isolates from 39 cases of dysentery and 30 isolates from 21 cases of control were included in this study. They were subcultured in 1.5 ml of trypticase soy broth containing 1 percent D-mannose and cultured at 37°C overnight.

Adherence cell assay test was done by using Lab Tek chamber slides seeded

with Hep-2 cells, a continuous line of human epithelial cells as described by Cravioto et al. [4]. These cells were grown at 37°C in 5 percent carbondioxide in Eagles minimal essential medium (MEM) supplemented with 100 U of penicillin/ml, 100 mcg of streptomycin/ml, 2 mM L-glutamine, 10 percent calf serum and 1 percent foetal calf serum until nearly confluent monolayer had formed.

After washing the slides with phosphate buffer saline three times, the cell culture medium was then replaced with MEM containing D-mannose without antibiotics.

0.05 ml of E. coli broth was then added, and the chamber slide was incubated at 37°C for 30 minutes (infection period). The slides were then washed five times and media with 1 percent mannose was added and again incubated at 37°C for 4 hour (multiplication period). The slides were then washed thoroughly five times with phosphate buffer saline. The cells were fixed with methanol, and stained with May-Grunwald stain diluted 1:2 with Sorensen buffer (pH 7.3) for 5 minutes and Giemsa stain diluted 1:3 with same buffer 20 minutes. The stained slides were washed with water, dried and examination was made under light microscope.

A strain was considered to be adherent if E. coli was observed to adhere to 40 percent of the Hep-2 cells. A known Enteroadherent Escherichia coli 0128 K67 (N 10/83) which was tested at CDC, Atlanta and E. coli ATCC 25922 were used as positive and negative controls respectively.

RESULTS

All E. coli isolates were serogrouped by using of antisera

produced in rabbits as according to the methods of Edwards and Ewing [11]. The EPEC serogroups sought were 01, 026, 044, 055, 086, 0111, 0114, 0125, 0126, 0127, 0128, 0142, 0146, 0157. The EIEC serogroups sought were 028, 0112, 0124, 0136, 0144. The ETEC serogroups sought were 06, 08, 025, 027, 0148 and 0159. Normal Hep-2 cell was shown in Fig. 1.



Fig. 1. Normal HEP-2 cells

When E. coli strains attach to Hep-2 cells, two attachment pattern occurs. In One, the bacteria are seen around or covering the whole surface of the Hep-2 cell (Fig. 2).



Fig. 2. Diffused adherence pattern in HEP-2 cells

This pattern is called diffuse adherence (DA). In the second kind of attachment, the bacteria bind to localized areas of Hep-2 cells in which they form very clear-cut

microcolonies (Fig. 3). This attachment pattern is called localized adherence (LA).



Fig. 3. Localized adherence pattern in HEP-2 cells

A total of 115 isolates of EPEC, 24 isolates of ETEC, 14 isolates of EIEC and 266 untypable isolates of *E. coli* were tested for adhesive assays. It was observed that out of 115 isolates of EPEC tested 20 isolates were showing adhesion (17.5 percent). When checking with different serogroups it was found that one isolate each of 01, 026, 0127 and 0142 showed adhesion respectively. Two isolates of 0125 also showed adhesion. Three isolates each of 055 and 086 were also showing adhesion. In serogroup 0128, 8 isolates were showing adhesion (Table 1). In EIEC serogroups out of 14 isolates tested no strain showed adhesion. In ETEC serogroups, one isolate of 08, 1 isolate of 027 and 2 isolates of 0159 showed adhesion respectively. A total of 4 isolates out of 24 cases of ETEC (16.7 percent) showed adhesion. 266 isolates of untypable *E. coli* (non EPEC, non ETEC) were tested and found that 22 isolates showed adherence. Type of adherence was shown in Table 2. In 20 isolates of EPEC it was found that 16 isolates showed diffused pattern and 4 isolates showed localized type of adhesion. In 4 isolates ETEC it was found that 2 isolates showed

Table 1. Distribution of Mannose resistant adhesion among EPEC, EIEC and ETEC serogroups

Serogroups	No. of isolate tested	No. (%) showing adhesion
EPEC 01	28	1 (3.6)
026	4	1 (25.0)
044	1	0
055	7	3 (42.9)
086	15	3 (20.0)
0111	1	0
0114	6	0
0125	12	2 (16.7)
0126	6	0
0127	5	1 (20.0)
0128	14	8 (54.4)
0142	11	1 (9.1)
0146	1	0
0157	4	0
all EPEC	115	20 (17.5)
EIEC 028	3	0
0112	5	0
0124	2	0
0136	2	0
0144	2	0
all EIEC	14	0
ETEC 06	4	0
08	4	1 (25.0)
025	5	0
027	5	1 (20.0)
0148	1	0
0159	5	2 (40.0)
all ETEC	24	4 (16.7)
UT	266	22 (8.3)

diffused pattern and 2 isolates showed localized pattern. In 22 isolates of non EPEC nor non ETEC (UT) group it was found that 10 isolates showed diffused patterns, 46 isolates showed localized pattern and 4 isolates showed aggregative type. It was also noted that out of 30 isolates from 21 control cases none of them showed adhesion.

Table 3 was showing the relationship

Table 2. Types of adherence pattern of some serotypes of E. coli

Serogroup	adherence			
	Total	Diffused	Localized	Aggregative
EPEC 01	1	1	0	0
026	1	0	1	0
055	3	1	2	0
086	3	3	0	0
0125	2	1	1	0
0127	1	1	0	0
0128	8	8	0	0
0142	1	1	0	0
Total	20	16	4	0
EIEC	0	0	0	0
EIEC 08	1	1	0	0
027	1	1	0	0
0159	2	0	2	0
Total	4	2	2	0
UT	22	10	8	4
Total	46	28	14	4

between adherence and type of infection. Out of 46 cases which were positive for Enteroadherent E. coli, 35 cases (20.2 percent) were from diarrhoea cases and 11 cases (28.2 percent) were from dysenteriae cases. These includes 20 cases of EPEC and found that 13 cases were from diarrhoea cases and 7 cases were from dysenteriae cases. In 4 cases of ETEC they were from diarrhoea cases. In 22 cases of non EPEC nor non ETEC groups it was observed that 18 cases were from diarrhoea and 4 cases were from dysenteriae cases.

DISCUSSION

Several studies have demonstrated that adherence to the intestinal mucosa is an important virulence factor of EPEC strains [1,12,13].

Table 3. Relationship between adherence and diarrhoea or dysenteriae

Serogroup	No. of cases positive	Diarrhoea (173 cases)	Dysenteriae (39 cases)
EPEC 01	1	1	
026	1	1	
055	3	3	
086	3	1	2
0125	2	1	1
0127	1		1
0128	8	5	3
0142	1	1	
Total	20	13	7
EIEC	-	-	-
EIEC 08	1	1	
027	1	1	
0159	2	2	
Total	4	4	
UT	19	15	4
Total	46	35 (20.2%)	11 (28.2%)

Other studies also suggested that Hep-2 cell adherence may be a good model of in vivo EPEC adherence [12,14,15]. Cravioto et al. [4] in the original description of Hep-2 cell adherence, reported that 29% of 17 non-EPEC, non ETEC strains of E. coli that were possible causes of five diarrhoeal outbreaks were adherent to Hep-2 cells. These reports strongly suggest that Hep-2 cell adherence may be a virulence factor in E. coli strains, independent of serogroup, and that EAEC strains may represent another diarrheagenic E. coli. Echeverria et al. [16] reported that well travellers to Thailand had a carriage rate of 13 percent of ETEC and in addition EAEC strains may account for a portion of 25 percent of cases of diarrhoea in which no agent can be identified. EAEC were isolated from 30.4 percent of these patients and these observations suggest an etiologic role of EAEC

strains in travellers diarrhoea.

Mathewson *et al.* [17] studied Hep-2 cell adherent *E. coli* strains most of which do not belong to EPEC serogroups, but may be of importance in diarrhoea in adults and reported that the EAEC strains isolated from adult travellers in their studies rarely belonged to traditional EPEC serogroups. They also reported that patients with diarrhoea in whom no recognized enteropathogen could be identified or had 30.4 percent prevalence of EAEC. Thus, conclusion was made that EAEC are associated with diarrhoea in travellers and suggest that Hep-2 cell-adherent *E. coli* strains, most of which do not belong to EPEC serogroups, may be of etiologic importance in travellers diarrhoea in adults.

Levine *et al.* [18] also studied on the diarrhoeal response of human to O127:H6 classic serotypes of EPEC and demonstrated that it is dependent on a plasmid encoding on enteroadhesive factor, which is located on plasmids 50-70 MDa in size.

Nataro *et al.* [19] detecting an adherence factor of EPEC by DNA and reported that O55, O142, O127, O111 and O119 showed adhesion. In our report also O55, O125, O126, O127, O128 and O86 showed adhesive pattern.

In our study it was also observed that in 266 isolates of non ETEC - non EPEC; 22 isolates showed enteroadherent (8.3 percent). No adhesion was occurred when tested in EIEC serogroups. 4 isolates of *E. coli* showed adhesion in 24 ETEC strains and 20 isolates out of 115 isolates of EPEC showed adhesion with different patterns of adhesion.

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