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Clinical trial of indigenous antipyretics : Testing of antipyretic efficacy of five traditional medicine formulations on TAB–induced pyrexial volunteers

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Two grams each of five traditional medicine formulations (TMF)- TMF-06, TMF-24, TMF-25, TMF-35A and TMF-35B- orally administered with 150 ml of betel leaf preparation, had underwent a clinical trial of antipyretic efficacy on a total of 315 TAB-induced pyrexial Burmese volunteers. Both positive control of acetyl salicylate 600 mg orally and negative control of no drug treatment were included. The trial design was performed on a single (observer) blind basis. Effectiveness of the agents at the peak of induced pyrexia were observed for three hours. Only TMF-06 possessed the antipyretic efficacy (78-88%) as could be observed in 87-96% of responders, in comparison to those of the acetyl salicylate. Nevertheless, TMF-24 and TMF-35B showed a lesser antipyretic activity. Therefore, TMF-06 was found to be the most recommendable antipyretics to be used at indigenous medicine centres.

INTRODUCTION

Fever is a very common affliction and many indigenous drugs are used extensively as antipyretics at the traditional medicine outpatient departments (1). However, none of them have ever undergone scientific scrutiny. The aim of this trial is to evaluate scientifically the antipyretic efficacy of traditional medicine formulations (TMF) namely TMF-06, TMF-24, TMF-25, TMF-35A and TMF-35B using acetyl salicylate as positive control on a single blind fashion.

MATERIALS AND METHODS

The formulations were supplied by the authorities of the Indigenous Medicine Council of the Department of Health Services. A total of 315 subjects both male and female of

ages between 12 to 50 years participated. The trial was conducted in three parts.

(1) Determination of method of fever induction

The temperature of each healthy subject was initially determined by taking three successive oral temperature with an interval of five minutes in between recordings. Their means were taken as basal temperature. Based on the fact that Salmonella typhosa endotoxin could induce pyrogenic reaction (2), five subjects were given 0.5 ml of TAB vaccine subcutaneously, another five were given 0.5 ml intramuscularly and the last five subjects were given 1.0 ml intramuscularly. Their changes in temperature were recorded. Only the group administered with 1.0 ml of TAB vaccine intramuscularly showed satisfactory fever induction and the induced pyrexia was found to be raised about 1.0° C lasting for five more hours.

(2) Investigation of own control applicability

The fever pattern was not reproducible in the same individual by the same method and therefore same subject serving as its own control could not be instituted.

(3) Main trial

The main trial was conducted on a total of 290 subjects, both male and female of ages between 12 to 50 years. The basal temperature of the subjects were first taken, then they were given 1.0 ml of TAB vaccine intramuscularly. The temperature was recorded at every thirty minutes for a total duration of eight hours. The subjects with satisfactory fever induction at the fifth hour were classified into high pyrexial responders (those with induced pyrexia of 1.0°C or more above basal level) and low pyrexial responders (those with induced pyrexia of 0.5° C to 0.9° C above basal level). Then the subjects were randomly allocated into two control groups and six test groups. The subjects in negative control group were not given any drug. The subjects in positive control group were given a single oral dose of 600 mg of acetyl salisylate together with 150 ml of betel leaf preparation which was prepared by boiling 0.5 g of betel leaves in 150 ml of boiled water for 5 mins. The subjects in the 5 test groups were given a single oral dose of 2 g of one of the test drugs with 150 ml of betel leaf preparation. The subjects in the last test group were given 150 ml of betel leaf preparation only.

The temperature in the six half-hourly observations following the oral administration of the drugs were noted and changes of temperature from that of the fifth hour were calculated. The mean temperature profile was illustrated graphically and the antipyretic efficacy was presented in a tabulated form.

Measures were taken to note any side effects during the trial and prompt and adequate treatment given as required.

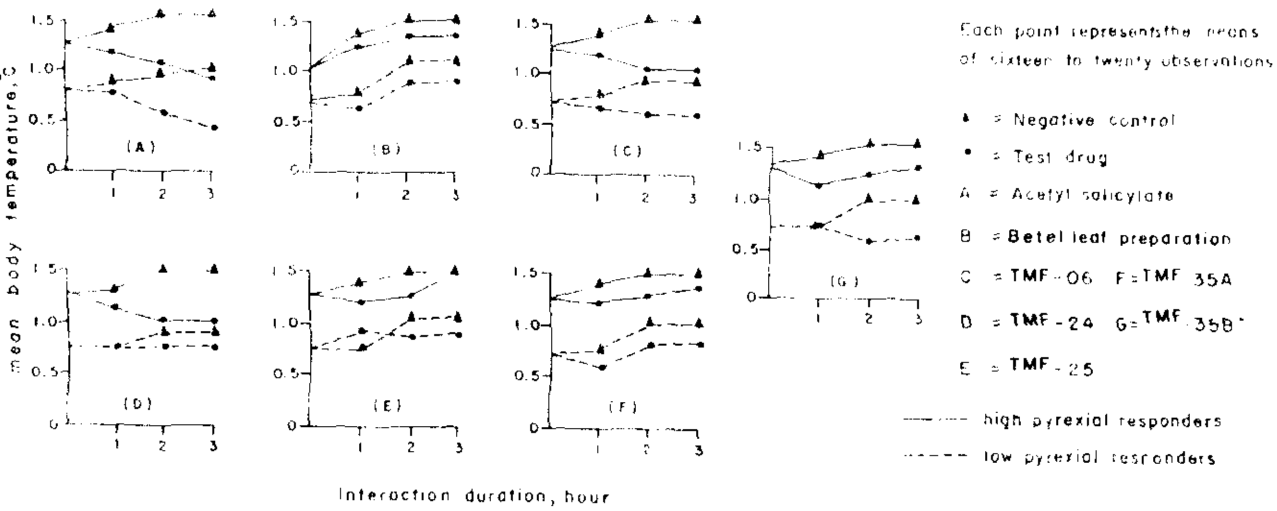

Fig. 1 - Mean temperature profiles of acetyl salicylate and test drugs after drugs intervention at the fifth hour in high and low pyrexial responders

RESULTS

The antipyretic efficacy of the six test drugs and acetyl salicylate in terms of temperature lowering capability are shown in Figure 1. Betel leaf preparation, TMF-25 and TMF-35A showed no significant antipyretic activity. Acetyl salicylate and TMF-06 showed significant antipyretic activity in 53% and 50% of subjects respectively in high pyrexial responders. In low pyrexial responders, acetylsalicylate , TMF-06 , TMF-24 , and TMF-35B respectively showed significant antipyretic activity in 61%, 53%, 40% and 37% of the subjects.

Compared to acetyl salicylate, the antipyretic efficacies of the various test drugs in order of merit were found to be as follows: TMF-06, TMF-24 and TMF-35B (Table 1).

Table [illegible] Temperature reducing capabilities of drugs with marked antipyretic activity

No	Drug	Temperature reducing capability					
		High pyrexial responders			Low pyrexial responders		
		Nett temperature reduction °C	efficacy %	Statistical significance student's 't' test	Nett temperature reduction °C	efficacy %	Statistical significance student's 't' test
1	Acetyl salicylate	[illegible]	100	p < 0.005	58	100	p < 0.005
2	TMF-06	[illegible]	88	p < 0.005	23	78	[illegible]
3	TMF-24	[illegible]	46	p < 0.025	[illegible]	[illegible]	[illegible]
4	TMF-35B	[illegible]	46	p < [illegible]	93	[illegible]	[illegible]

DISCUSSION

This trial established the method of fever induction in testing drugs with reputed antipyretic effects. It also showed that the subjects cannot be used

as its own control. In this trial all six test drugs exhibited antipyretic activity. However, only TMF-06, TMF-24 and TMF-35B exhibited a statistically significant antipyretic activity, out of which TMF-06 was most efficacious either in terms of percentile respondence or temperature lowering capability.

REFERENCES

1. Indigenous practitioners society, Drugs Used by the Burmese Traditional Medicine, Indigenous Medical Education, Ministry of Health, Burma. (Burmese version) 1982;21-67.

2. Greisman,S.E and R.B.Hornick Comparative pyrogenic reactivity of rabbit and man to bacterial endotoxin.Proceedings of the Society for Experimental Biology 1969; 131 (1) : 1154-1158.

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Application of modified Enzyme-Linked Immunosorbent Assay (ELISA) for detecting virulent strains of enteroinvasive *E.coli* and *Shigella*

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A virulent isolate of Shigella dysenteriae 1 strain, HW 829 was used for the production of specific antibody. Three rabbits (Japanese White strain) were immunized and the serum was obtained. These sera were absorbed with a virulent derivative of the same strain (i.e. HW 829). Tube agglutination titer of immune sera was varied from 1:1,280 to 1;5,120 with living and 1:5,120 to 1:20,480 with boiled cells of the homologous strain. The absorbed sera were utilized as an antibody in ELISA assay using BIOTIN kit. The investigation of 33 Shigella and 22 enteroinvasive E. coli (EIEC) strains indicated complete agreement between ELISA results and those of the other virulence tests (Sereny test and HEp-2 tissue culture assay).

INTRODUCTION

The invasive potential of Shigella and enteroinvasive E. coli (EIEC) is increasingly recognized as an important pathogenic factor (1). Three methods for testing the invasive potential of enteroinvasive microbes are commonly used nowadays. These are production of dysentery in primates, Sereny test (2) and in vitro tissue culture test (3) using sensitive cell lines. As these methods for the study of invasive capabilities are applicable to individual isolates only, a rapid and economical test is needed for the routine use and epidemiological studies. In this paper, how a modified ELISA test has been introduced to identify virulent Shigella and IEC strains as a routine test was reported.

METERIALS AND METHODS

Clinical isolates of 54 E. ccli strains, 33 Shigella and 14 strains of Salmonella were included in the study. Among the E. coli strains, 22 were enteroinvasive, 9 were enterotoxigenic and 23 were enteropathogenic according to biochemical and serological identification methods of Edward

and Ewing (4). Kereto conjunctivetis test (Sereny test) was performed according to the method of Sereny. And HEp-2 tissue culture assay was done in order to check the invasive property as mentioned by Day et al. (3).

Production of antibodies - A virulent isolate of Shegella dysenteriae 1 named HW 829 was used for the production of specific antibodies. This strain was kindly provided by National Institute of Health, Tokyo, Japan. Three rabbits were immunized intravenously at 4-day interval with 0.2, 0.5, 1.0 and 2.0 ml respectively, of a suspension containing 10 living bacteria per ml. Blood was obtained on day 4 after the last injection. Tube agglutination titers of the three immune sera varied from 1:1,280 to 1:5,120 with living and 1:5,120 to 1:20,480 with boiled cells of the homologous strains.

These sera were absorbed with nonvirulent, Sereny-negative derivative of HW 829 strain, which was obtained by serial passages on nutrient agar at 42° C. The resultant sera did not show any agglutination with both live or boiled cells of HW 829 strain. Sera were membrane-filtered and stored at -80° C without preservation.

ELISA Assay - Wells of Dynatech Micro-Elisa plates were sensitised overnight at 4° C with 10 bacteria suspended in 100 ul of coating buffer (Na_2CO_3 1.59g, $NaHCO_3$ 2.93 g in 1000 ml of distilled water, pH 9.6). 100 ul of rabbit immune sera diluted in PBS-Tween 20 was added, followed by 1 h incubation at 30° C. Then 100 ul of conjugate (anti-rabbit IgG labelled with horse raddish peroxidase (Vestestain, ABC kit, Vector laboratory, Inc) diluted 1:2,000 in PBS-Tween containing 0.5% BSA was added.

After further incubation for 1 h at 30° C, the reaction was developed by adding 150 ul of the substrate (0-phenylenediamine-dihydrochloride in citric acid buffer, pH 6.0). The reaction was stopped after 10 minutes by adding 50 ul of strong sulphuric acid.

In each experiment, strains were tested in three parallels. Optical density (OD) was measured at 492 nm with Dynatech ELISA reader. Wells without bacteria served as blanks. Additional controls were wells with normal rabbits serum and wells without rabbit immune serum. The blanks were used to set the photometer at O.

RESULTS

Optimization of the ELISA test - ELISA reactivity of one of the three antisera was tested against six pairs of Sereny-posiitive and -negative Shigella and E. coli (enteroinvasive strains). Different dilutions of antisera were used in the ELISA test. OD values are shown in Table 1. Antisera rected with the Sereny-positive strains at a remarkably higher dilution than did with the negative strains regardless of serogroups. In further studies, antisera were used in a dilution of 1:1,000.

Table 1. Correlation between virulence and reactivity of antisera at different dilutions in ELISA test.

Strains	Sereny test	OD values in dilutions 1:200	1:500	1:1,000	[illegible]
Hm 829 (virulent)	+	1.63	0.97	0.[illegible]	.Hm
Hm 8[illegible] (cured)	-	0.04	0.00	0.00	0.00
E.coli 0 [illegible]	+	1.33	0.98	0.90	0.[illegible]
	-	0.00	0.00	0.00	0.00
E.coli 0 136	+	0.98	0.92	0.86	0.76
	-	0.00	0.00	0.00	0.00
E.coli 0 144	+	1.06	0.99	0.92	0.84
	-	0.00	0.00	0.00	0.00
E.coli 0	+	1.50	1.22	1.12	1.00
		0.02	0.03	0.00	0.00
E.coli 0 164	+	1.02	0.92	0.92	0.80
	-	0.00	0.00	0.00	0.00

Reactivity of antisera with strains of different origin - Thirty-three Shigella and 22 E. coli strains were examined by this method. The strains that were positive by Sereny test reacted strongly with the antisera(OD range 0.36 to 1.5) (Table 2). For these strains the mean OD value was 0.96 $\pm$ 0.28 standard deviation. Countrary to this, Sereny-negative strains had a low OD value (range 0.00 to 0.3) with a mean of 0.7$\pm$0.005 standard diviation.

According to these values a strain was considered as positive when the OD value exceeded 0.32 (the mean -2 SE for the Sereny-positive strains). The difference between the OD values of the two groups was found to be significant at p=0.0001 by the Student´s ´t´ test. At this dilution antisera gave a clearcut differentiation between virulent and non-virulent isolates and the antisera did not react with the other enteric pathogens like ETEC, EPEC or Salmonella spp. This feature was so pronounced that positive strains could be identified at the wells by the naked eyes.

Table 2. Comparison between virulence and antibody reactivity.

Strains	No. of strains virulence	tested	ELISA positive	negative	Range
Shigella.dysen	+	27	27	0	0.[illegible]-1.36
	-	3	0	3	.14-0.28
S.flexneri	+	5	5	0	0.[illegible]
S.sonnei	-	2	0	2	.34-0.3
E.coli 0 26	+	3	3	0	0.44-0.72
0 111	-	1	0	1	0.20
0 124	+	3	3	0	0.6[illegible]-1.46
0 136	+	4	4	0	0.36-1.24
	-	4	0	4	0.0[illegible]-0.[illegible]
0 144	+	1	1	0	1.50
0 152	+	2	2	0	[illegible]-[illegible]
	-	1	0	1	0.[illegible]
0 164	+	3	3	0	0.56-0.8[illegible]
Total positive	+	44	44	0	0.36-1.5[illegible]
Total negative	-	11	0	11	0.00-0.3[illegible]

Virulence was estimated by the method of Sereny and HEp-2 tissue culture tests.
Range of OD means values as estimated by three parallel wells at 492 nm.

DISCUSSION

The determination of virulence of invasive E. coli and Shigella strains has both clinical and epidemiological importance. The recent methods for the study of invasive properties are applicable only to individual isolates. These methods are not suitable for identification of a large number of isolates. A rapid and economical test is needed for epidemiological purposes.

On the basis of results for 44 sereny-positive and 11 sereny-negative strains of different

serogroups, it appeared that specific antisera reacts with a unique antigenic moiety expressed by the enteroinvasive strains.

When the virulency of the strains was checked by using *in vitro* tissue cluture test, all the sereny-positive strains show invasion into the HEP-2 cells.

These results suggested that virulent strains of EIEC and *Shigella* had similar or identical antigens which is absent in the virulent strains (Table 3) and other enteric pathogens like EPEC, ETEC and Salmonella spp. (Figure 1). Therefore, the present ELISA technique is suitable to test efficiently large numbers of strains or colonies within 24 hours. Further improvement of the test to detect antibody directly from fecal samples is necessary.

Table 3. Comparison of ELISA to other virulence tests

Strains	No. tested	ELISA	Sereny	HEp-2 cell invasion
E.coli	16	+	+	+
	6	-	-	-
Shigella	28	+	+	+
	5	-	-	-

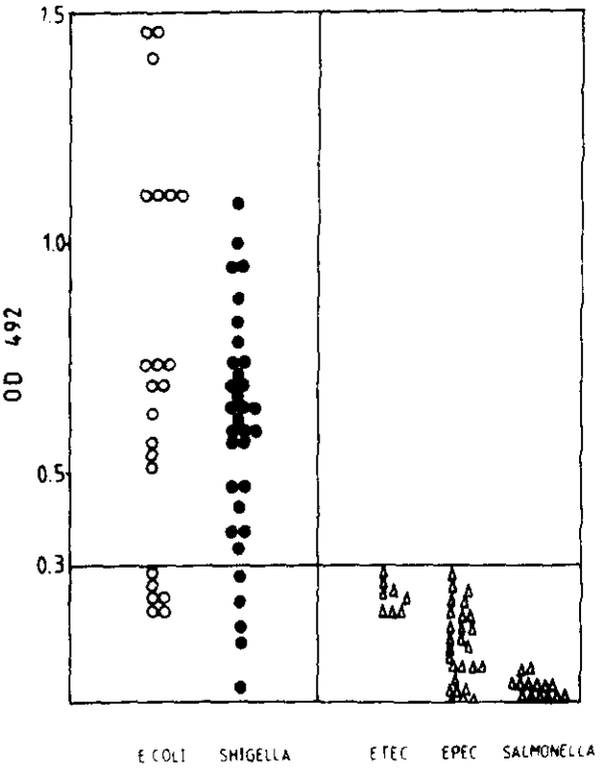

Figure 1. Reaction of antisera to virulent and avirulent *Shigella*, *E.coli* (EPEC, ETEC and EIEC) and *Salmonella* spp.

REFERENCES

1. Labrec,E.H., Schneidec,H.,and Formal,S.B. *et al*. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentry. *Journal of bacteriology* 1964;88:1503-1518.

2. Sereny , B. Experimental kerato conjunctivitis shigellosa. *Acta Microbiologica Academic Scientia* (Hungary) 1956;4:367-377.

3. Day,N.P., Scotland,S.M., and Rowe, B. Comparison of an HEp-2 tissue culture test

with the Sereny test for detection of Emteroinvasiveness in Shigella spp. and Escherichia coli. Journal of clinical Micrology 1981; 13 (3):569-597.

4. Edward, P.R. and Ewing, W.H. (Eds).Identification of Enterobacteriaceae. Burgess, Minneapolis, 67-142.