CHAPTER IV

RESULTS

1. The volunteers and types of vaccination

The clinical history of the volunteers is clearly an important aspect of this study. Before they were selected each was subjected to stool cultures which were examined particularly for S.typhi (group D).

All of the the volunteers used in this study were shown not to be carriers of typhoid fever and their sera were negative or < 1:20 (the normal range value) for the O and H antigens of S.typhi. Further' no volunteer has been previously vaccinated either parenterally or orally with a typhoid vaccine, at least, for the last three years.

Table 1 shows the ages and sexes of the volunteers, the type of vaccine they received and the samples taken for them.

2. Stool examination after vaccination

As S.typhi Ty 21a is an auxotrophic mutant of the parent pathogen S.typhi Ty 2 which causes typhoid

Table 1 Twenty-volunteers were devided into two groups by the types of oral vaccines.

group	vaccine	No.of volunteers		Age	Samples
		male	female		
1	Vivotif ®	5	6	38±2.48 * (26-51)	blood serum saliva
2	Thai Red Cross	6	3	25±2.12 * (18-37)	stool lavage

* = mean ± S.E.

fever, it was essential to check that there was no reversion to the wild-type following oral administration of Ty 21a.

We therefore performed stool examinations on the 1 day after each volunteer has received the oral vaccine. The results of these examinations showed the total absence of the wild-type strain, Ty 2.

3. The volume, input and output of isotonic solution

In order to determine the intestinal IgA in human volunteers after vaccination with Ty 21a, an intestinal lavage system was used, (as described in chapter III, section 4).

The data presented in Table 2 is given to show the average input volume of isotonic solution and average output volume of intestinal lavage with the Vivotif ® and Thai Red Cross groups, respectively.

4. The optimal conditions in the ELISA method used

4.1 The Determination of the activity and working dilution of rabbit anti-human-α-chain conjugate alkaline phosphatase

Rabbit anti-human- α -chain was, coupled to alkaline phosphatse by one-step glutaraldehyde technique as described in chapter III, section 5.2. This conjugate was

Table 2 Comparison of the volume of isotonic solution input [a] and output [b]

group	No. of volunteers [c]	No. of experiments [d]	type of vaccines	volume of isotonic solution [e]	
				input	output
1	6	42	Vivotif®	2103±99.0	364±14.4
2	9	63	Thai Red Cross	2714±90.0	377±9.8

a = The volume of isotonic solution administerd to the volunteers

b = The volume of intestinal lavage obtained from volunteers

c = person

d = intestinal lavage was performed seven times on each volunteers ; before and after vaccination at 1,2,3 weeks, 1, 2 and 3 months

e = Mean volume ± S.E. of all experiments

assessed by reacting which purified human IgA (Sigma).

The data in Figure 8, showed that the working dilution of our conjugate had good activity at 1:500 when incubated for 30 minutes and 60 minutes, but that long incubation gave a high background.

4.2 The Determination of the activity and working dilution of rabbit anti-human-α-chain urease conjugate

The procedure to determine the rabbit anti-human- α -chain urease conjugate activity was similar to tha for alkaline phosphatase conjugate (above). We found that the rabbit anti-human-α- chain urease conjugate had a high activity at 1:100. The end point is a colour change from yellow to deep violet.

4.3 Determination of the Optimal Concentration of CLPS for coating the polystyrene plates

As illustrated in Figure 9, we found that the optimal concentration for CLPS of S.typhi to interact at the carrier surface was 5 µg/ml similar to the results of Carlsson and co-workers (112), so that in the later work we used 5 µg/ml of CLPS of S.typhi to coat plates when determining specific IgA.

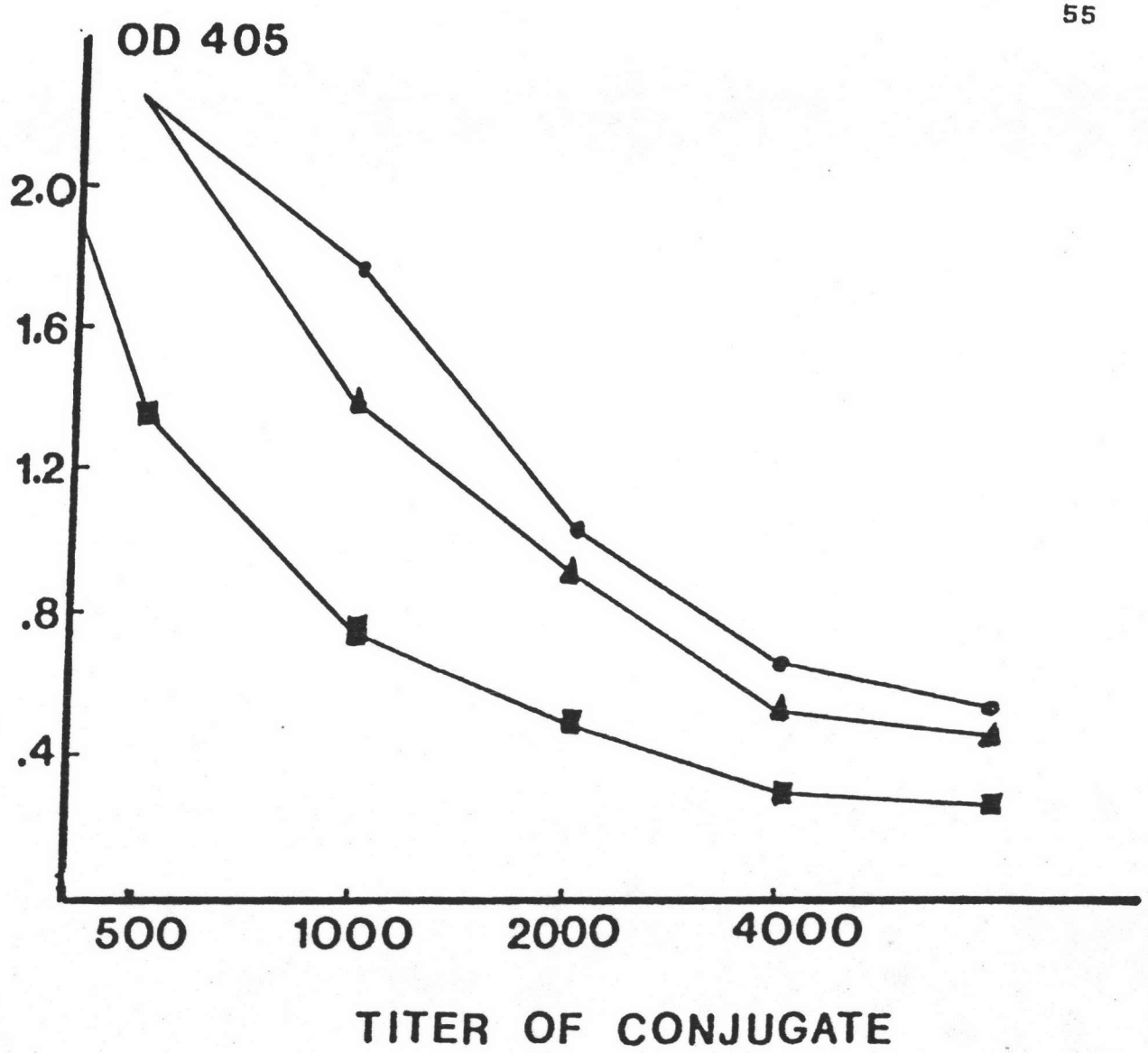

Figure 8 The determination of the activity and working dilution of rabbit anti-human- α -chain conjugate alkaline phosphatase; serial dilutions of the conjugate were added to polystyrene plates previously sensitized with purified human IgA (200 µl at 5 µg/ml). The plates were incubated at 37 °c for 3 hrs, washed 3x with PBS-Tween prior to the addition of suitable dilutions of conjugate. After 3 hrs at 37 °c of incubation the chromogenic substrate was added to each well of the whased plates. The incubation period 37 °c for 15 mins (■), 30 mins (▲) and 60 mins (●).

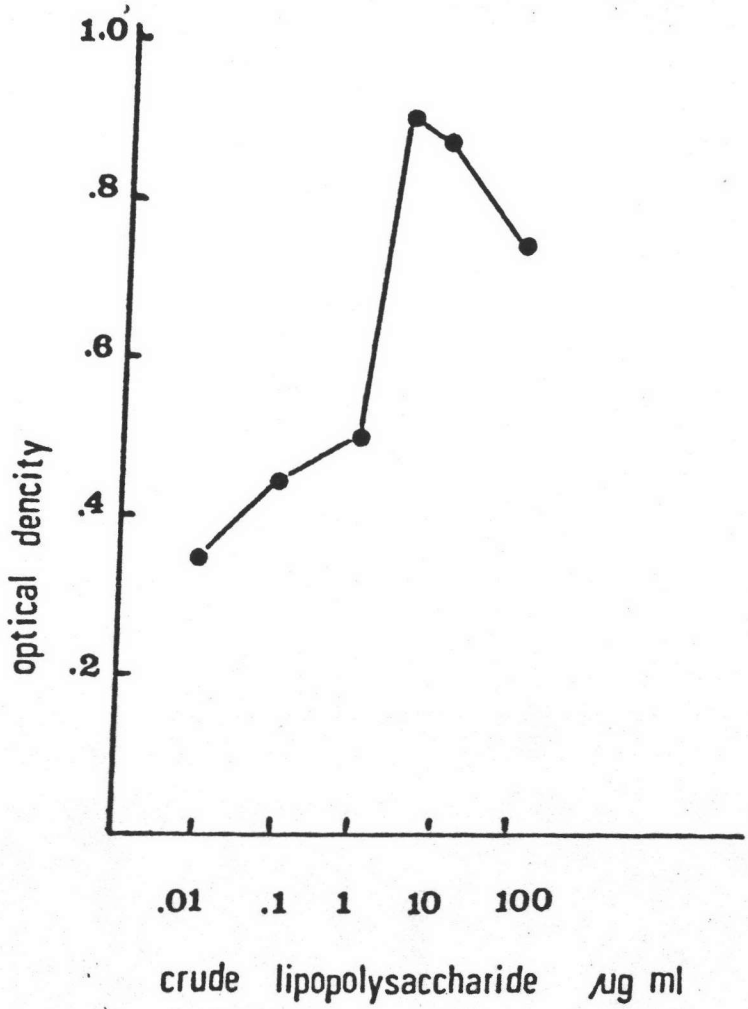

Figure 9 Determination of optimal concentration of CLPS for coating polystyrene plates ; varying amounts of S.typhi CLPS were used to coat plates and then positive antiserum from typhoid pateints were diluted at 1:100 and 200 µl of them incubated with the various CLPS coated plates. Rabbit anti-human- α -chain alkaline phosphatase conjugate(1:500) was added and then substrate was incubated at 37 ° c for 30 minutes.

4.4 The Optimal Temperature and Time for incubation of the samples

The optimal binding IgA from serum, saliva, stool extract and intestinal lavage to the CLPS coat was determined as follows :

4.4.1 Serum Ig A: As illustrated in Figure 10, the binding of serum IgA to the CLPS of S.typhi was virtually complete after four hrs, at 37 °c. Detachment will occur at the times incubation exceeding five hours.

4.4.2 Saliva Ig A: As illustrated in Figure 11, the incubation of saliva IgA anti-CLPS of S.typhi was virtually complete after 18 hrs at 25 °c.

4.4.3 Intestinal Lavage IgA : As illustrated in Figure 12, the incubation of intestinal lavage IgA anti-CLPS of S.typhi was virtually complete after 18 hrs, at 25 ° c.

4.4.4 Stool Extract IgA: As illustrated in Figure 13, the incubation of stool extract IgA anti-CLPS of S.typhi was virtually complete after 1 hr and at 4 ° c of incubation.

4.5 The Incubation with Conjugate

The optimal condition for the interaction of the rabbit anti-human- α -chain alkaline phosphatase conjugate was determined. As illustrated in Figure 14, that

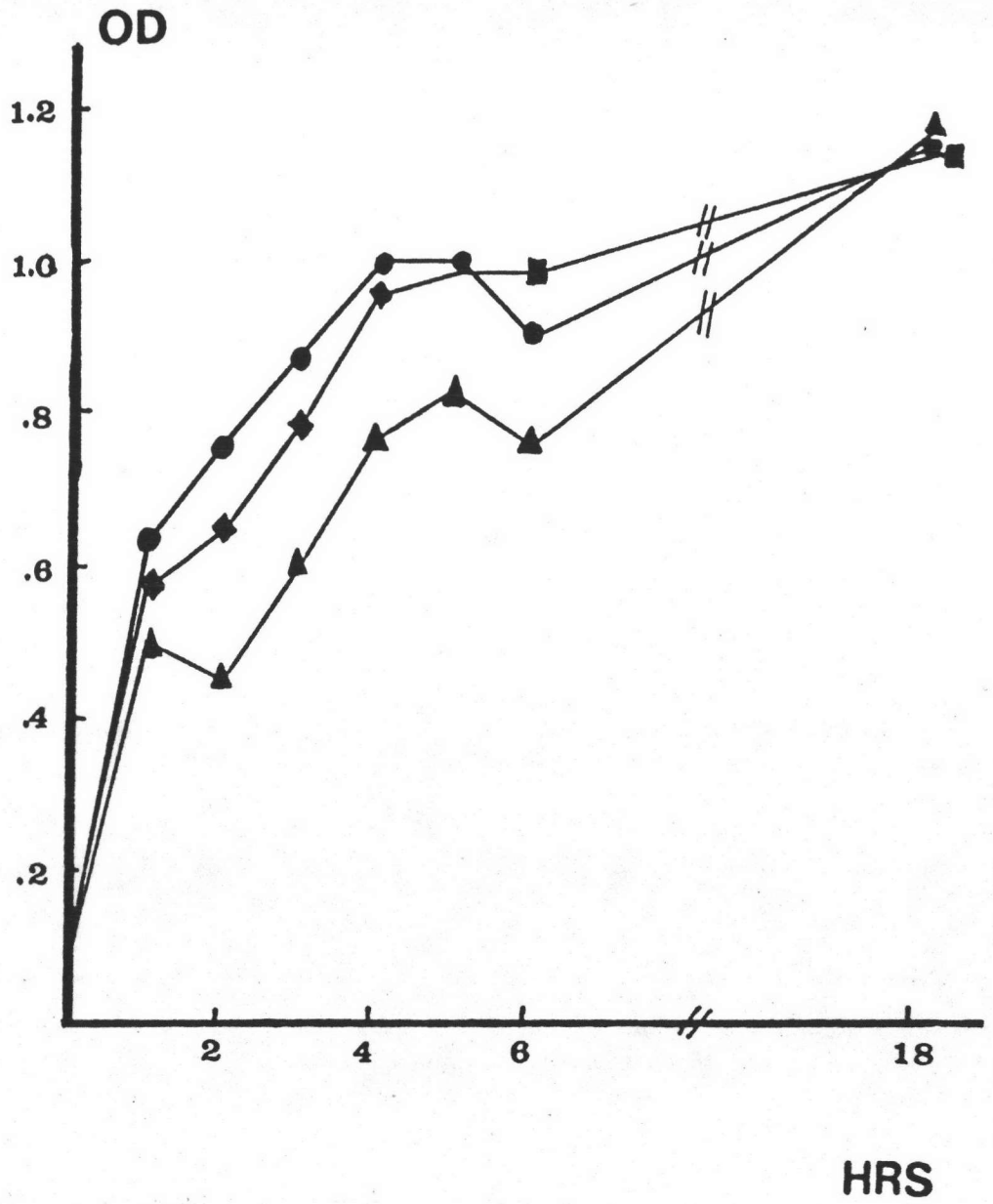

Figure 10 Kinetics of IgA in serum binding to CLPS coated plates; the microtiter plates were coated wiith CLPS (200 µl at 5 µg/ml)and incubated at 25 ° c for 18 hrs. The positive antiserum from typhoid pateints was diluted at 1:500 and separate incubation at various times and various temperatures,37 °c (●),25 °c(■) and 4 °c (▲) were carried out. The antibody binding was estimated as bound alkaline phosphatase conjugate to rabbit anti-human- α -chain(1:500) at 25 ° c for 18 hrs.

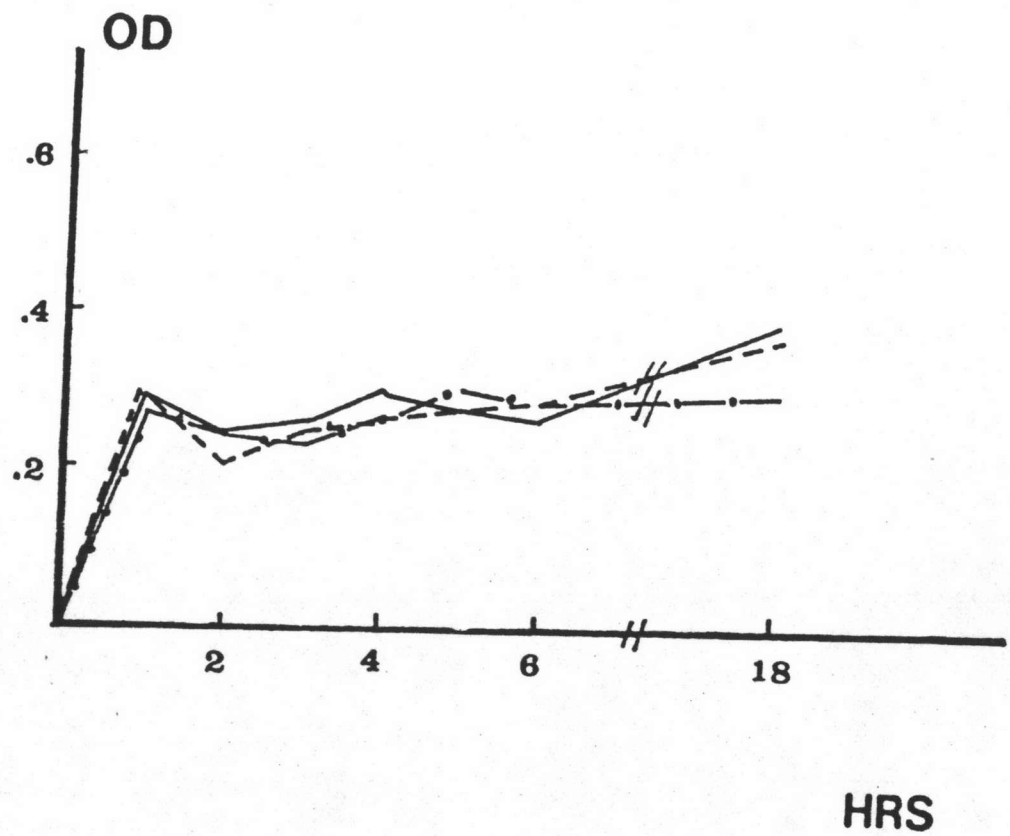

Figure 11 Kinetics of IgA in saliva binding to CLPS coated plates; The microtiter plates were coated with CLPS (200 µl, at 5 µg/ml), and incubated at 25 °c for 18 hrs. The saliva from vaccination volunteers were diluted at 1:40 and separated incubation at various times and various temperatures, 4 ° c (←–), 25 ° c (——) and 37 °c (←·–·–). The antibody binding was estimated as bound alkaline phosphatase conjugate to rabbit anti-human- α-chain(1:500) at 25 °c for 18 hrs.

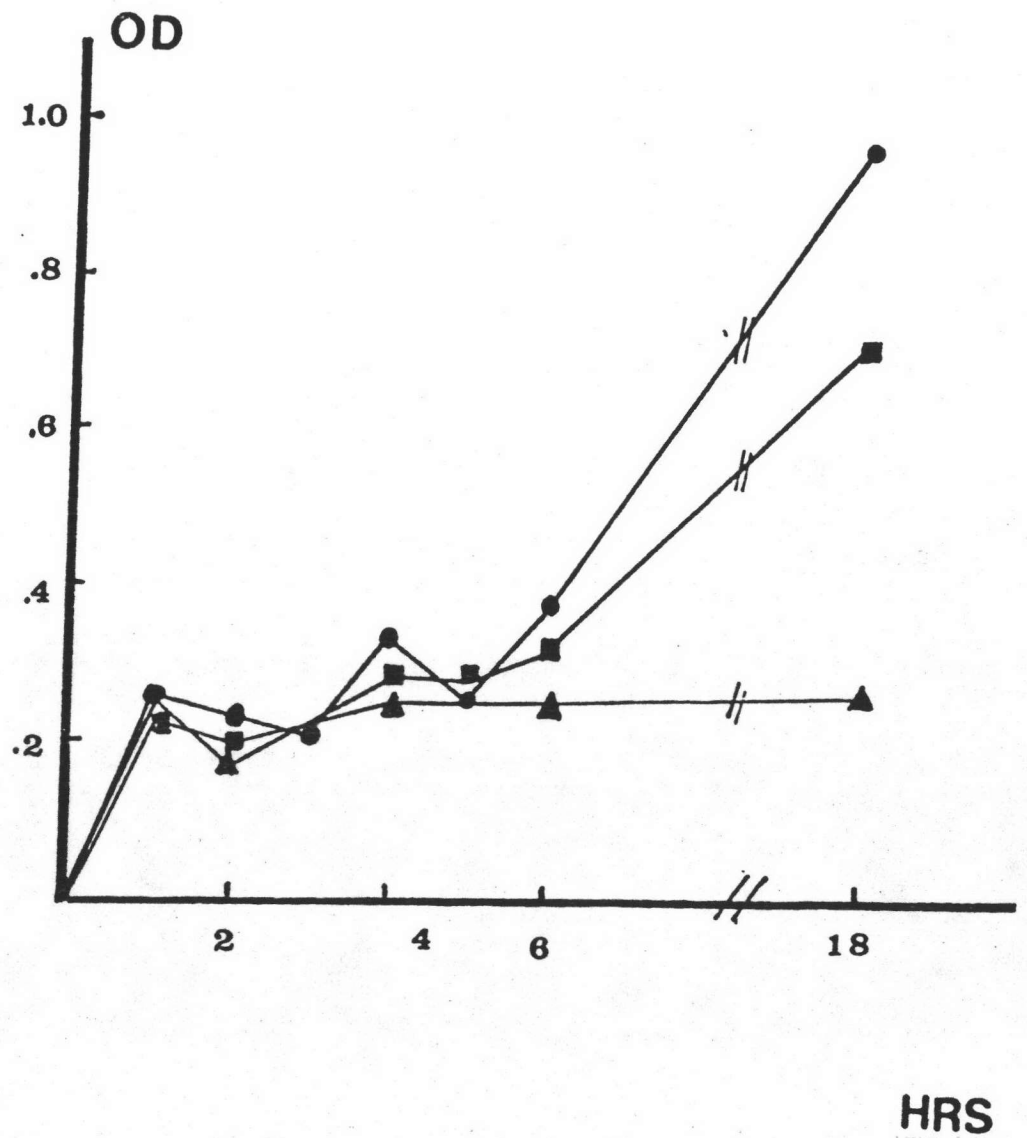

Figure 12 Kinetics of IgA in intestinal lavage to CLPS coated plates; the microtiter plates were coated with CLPS (200 µl, at 5 µg/ml), and incubate at 25 ° c for 18 hrs. The intestinal lavage from vaccinated volunteers were diluted 1:100 and separated incubation at various times and various temperature, 4 °c (■), 25 ° c(●) and 37 °c (▲). The antibody binding was estimated as bound alkaline phosphatase conjugate to rabbit anti-human-α-chain (1:500) at 25 ° c for 18 hrs.

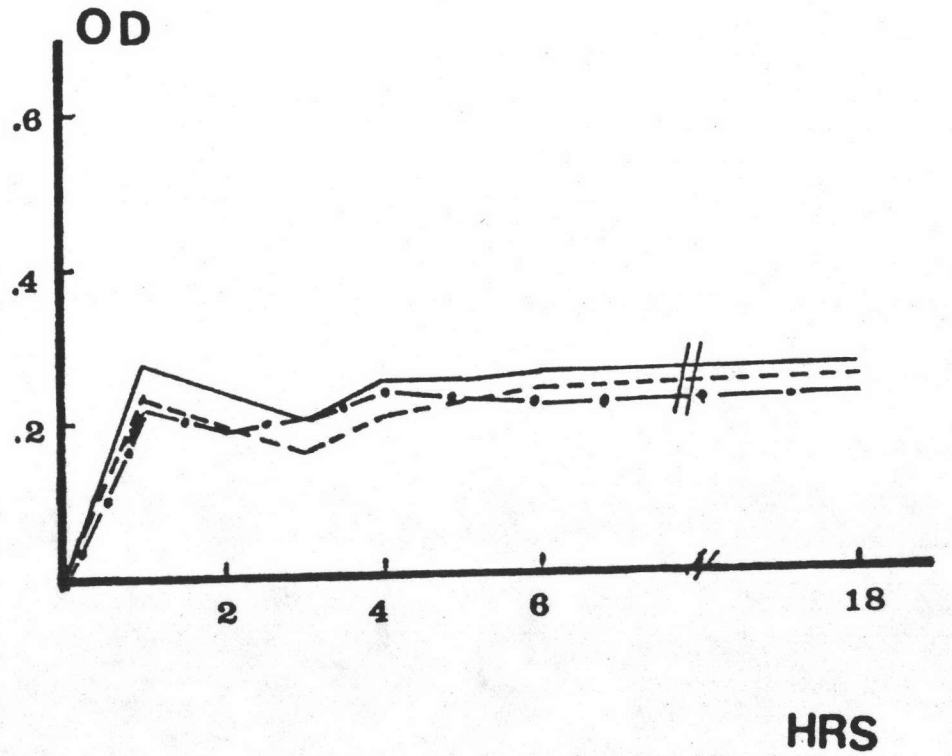

Figure 13 Kinetics of IgA in stool extract binding to CLPS coated plates; the microtiter plates were coates with CLPS (200 µl, at 5 µg/ml), and incubated at 25 ° c for 18 hrs. The stool extract from vaccinated volunteers were diluted at 1:20 and separated incubation at various times and various temperature, 4 ° c (—), 25 ° c (-•-•-), 37 ° c (- - -). The antibody binding was estimated as bound alkaline phosphatase conjugate to rabbit anti-human- α -chain(1:500) at 25 ° c for 18 hrs.

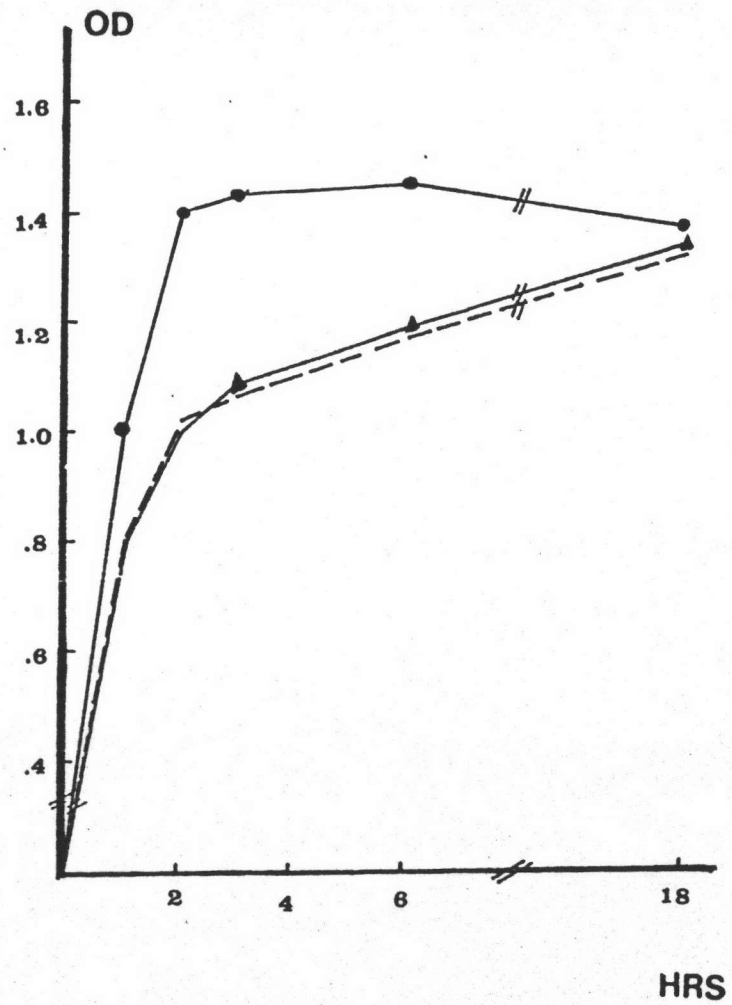

Figure 14 Kinetics of the rabbit anti-human- α -chain alkaline phosphatase conjugate binding; microtiter plates were coated with CLPS(200 µl, at 5 µg/ml)),and incubated at 25 ° c for 18 hrs. Then the positive antiserum from typhoid pateints was diluted at 1:50 and added to each well. After washing the fixed concentration of rabbit anti-human-α-chain alkaline phosphatase conjugate (1:500) were incubated at various times and various temperatures, 4 °c(---),25 °c(▲), and 37 °c (●). Bound conjugate was estimated as enzyme activity after 30 minutes.

the maximum binding of conjugate required about 2 to 3 hrs at 37 ° c.

As a result of the these findings,the ELISA protocols summarized in Table 3 and Table 4 were devised for use in these studies.

5. The leukocyte migration inhibition index

The migration inhibition index to CLPS antigen from peripheral blood before and after vaccination with typhoid vaccine is shown in Table 5,Figure 15 and Figure 16.

It was found that in both groups of vaccinees, the LMI indices were normal (LMI index = 0.8) before vaccination and were slightly inhibited (0.65-0.75 LMI index) but significant ($p < 0.05$) at 4 weeks through 12 weeks after vaccination versus control (0 week).

6. The serum IgA anti-CLPS response

The IgA response to CLPS antigen from serum was showed in Table 6, Figure 17 and Figure 18.

It was found that in both groups of vaccinees, the concentration of serum IgA after vaccination, was not significantly increased ($p > 0.05$) versus the control (0 week).

Table 3 ELISA Methods : Procedure for Determination of specific IgA anti-CLPS by alkaline phosphatase

	Step	Diluent	Concentration	Volume/well ul	Incubation time	Incubation temperature
1.	Precoat plates with crude LPS	carbonate bicarbonate	5 ug/ml	200	18 hrs	25 °c
2.	Wash 3x with PBS-Tween	-	-	200	-	-
3.	Add test samples :					
	3a. serum	PBS-Tween	-	200	4 hrs	37 °c
	3b. saliva and lavage	"	-	200	18 hrs	25 °c
	3c. stool	"	-	200	1 hr	4 °c
4.	Wash 3x with PBS-Tween	-	-	200	-	-
5.	Add AP labelled rabbit anti-human- α -chain	Triethanolamine	1:500	200	2 hrs	37 °c
6.	Wash 3x with PBS-Tween	-	-	200	-	-
7.	Add enzyme substrate (p-nitrophenylphosphate)	Diethanolamine	1 mg/ml	200	30 mins	37 °c
8.	Stop reaction with NaOH	-	3 M	25	-	-
9.	Read O.D. at 405 nm in spectrophotometer					

Table 4 ELISA methods : Procedure for determination of specific serum IgA anti-CLPS by urease conjugate

Step	Diluent	Concentration	volume/well ul	Incubation Time	Incubation Temparature
1. Precoat plate with crude LPS	Carbonate-bicarbonate	5 ug/ml	200	18 hrs	25 ° c
2. Wash 3x with PBS-Tween	-	-	200	-	-
3. Add serum test sample in serial dilution	PBS-Tween	-	200	4 hrs	37 ° c
4. Wash 3x with PBS-Tween	-	-	200	-	-
5. Add urease labelled rabbit anti-human-α chain	Triethanolamine	1: 100	200	18 hrs	25 ° c
6. Wash 3x with PBS-Tween	-	-	200	-	-
7. Wash 2x with D.W. *	-	-	200	-	-
8. Add substrate (BCP-Urea)	-	-	200	30 mins	37 ° c
9. Stop reaction with merthiolate	-	1 %	25	-	-
10. Read by visual detection yellow to violet					

* very important for urease

Table 5 Leukocyte migration Inhibition (LMI) index [a] in vaccinees who received two different typhoid vaccines

Time	Vivotif ®	Thai Red Cross
0 week	0.97 ± 0.06	0.96 ± 0.07
1 week	0.96 ± 0.09	0.85 ± 0.08
2 weeks	0.89 ± 0.09	0.88 ± 0.09
3 weeks	0.91 ± 0.08	0.83 ± 0.08
4 weeks	0.74 ± 0.06[b]	0.74 ± 0.04[b]
8 weeks	0.75 ± 0.05[b]	0.76 ± 0.02[b]
12 weeks	0.73 ± 0.06[b]	0.76 ± 0.04[b]

a = Mean ± S.E. of 11 and 9 vaccinees in Vivotif ® and Thai Red Cross groups, respectively.

b = $p < 0.05$ versus control (0 week) by student's t test.

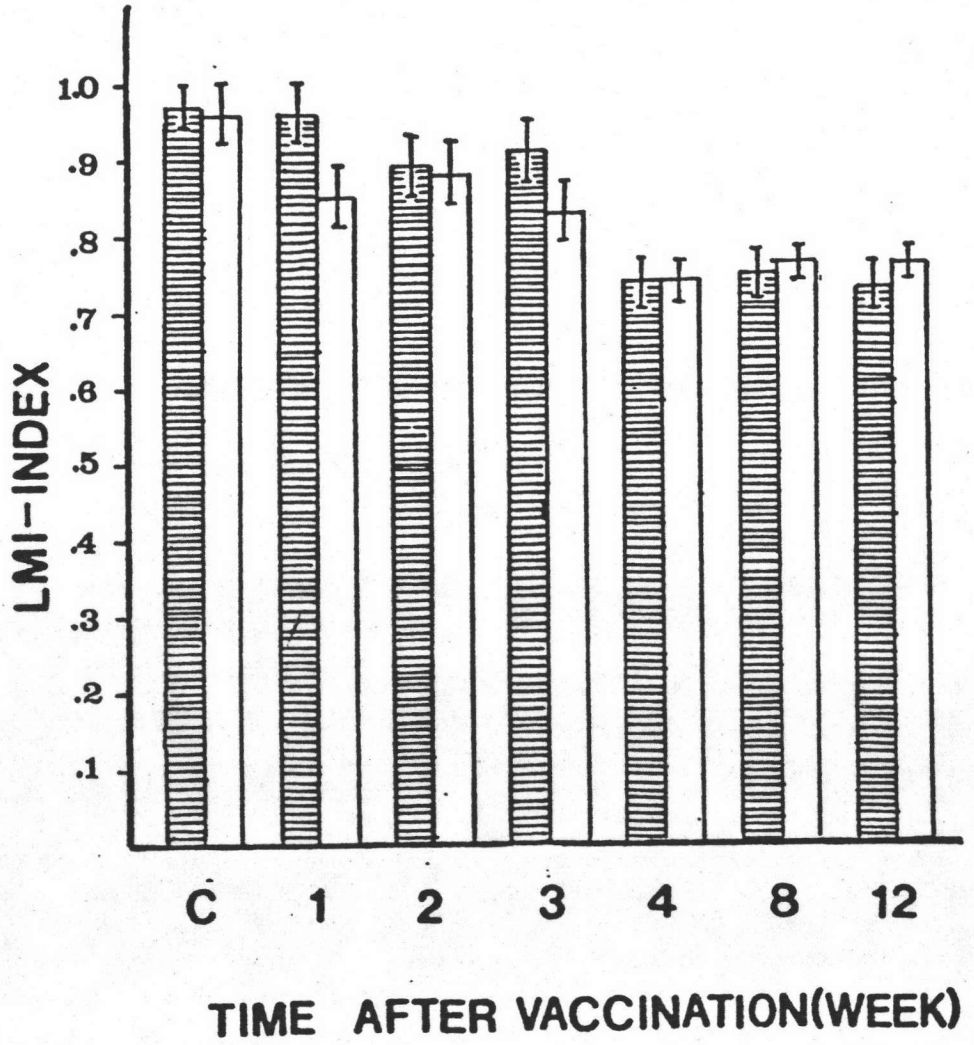

Figure 15 The specific cell-mediated immune response CLPS antigen by the measurement of LMI index before and after vaccination with Ty 21a vaccine.

▤ = Vivotif ® , □ = Thai Red Cross

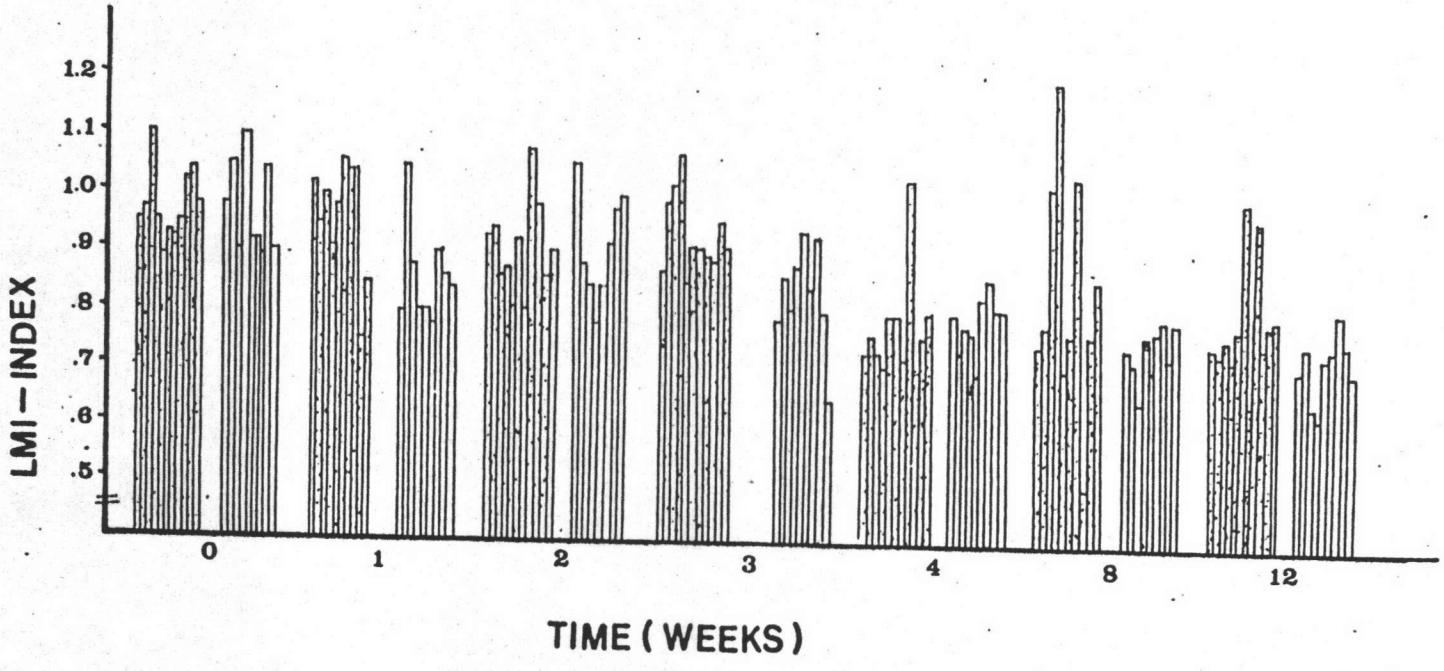

Figure 16 The individual LMI-index of 11 and 9 vaccinees in Vivotif ® and Thai Red Cross groups, respectively.

▨ = Vivotif ® , □ = Thai Red Cross

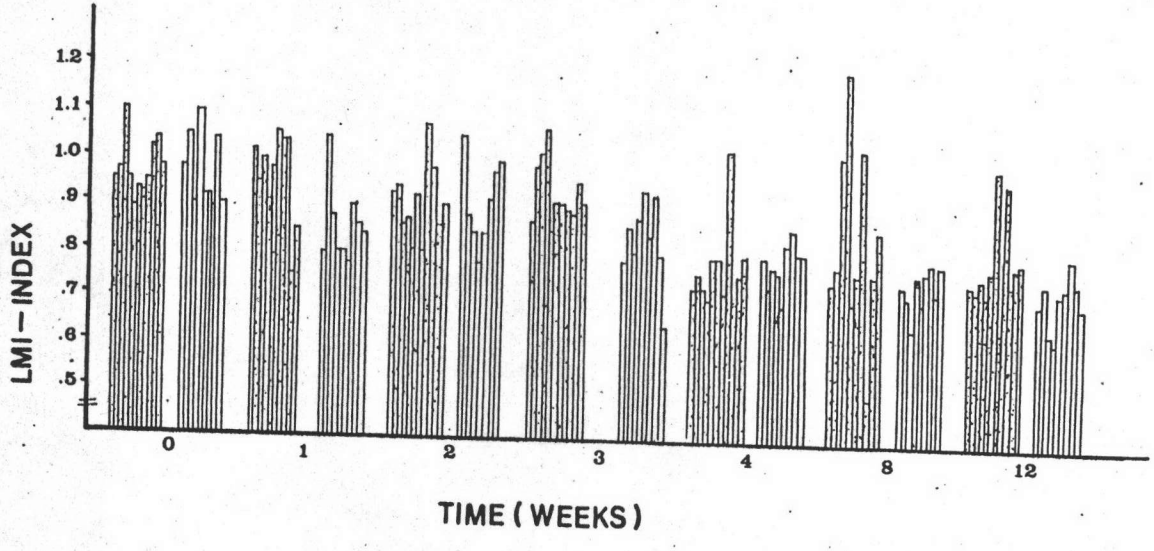

Figure 16 The individual LMI-index of 11 and 9 vaccinees in Vivotif ® and Thai Red Cross groups, respectively.

▨ = Vivotif ®, □ = Thai Red Cross

Table 6 The Systemic serum IgA anti-CLPS in vaccinees who received two different typhoid vaccines.

Time	Vivotif ®	Thai Red Cross
0 week	375 ± 296	163 ± 50
1 week	483 ± 287[b]	221 ± 160[b]
2 weeks	352 ± 284[b]	175 ± 54[b]
3 weeks	400 ± 184[b]	150 ± 52[b]
4 weeks	391 ± 273[b]	175 ± 54[b]
8 weeks	483 ± 258[b]	175 ± 85[b]
12 weeks	583 ± 278[b]	221 ± 47[b]

a = Geometric mean titer ± S.E. of 11 and 9 vaccinees in Vivotif ® and Thai Red Cross groups, respectively.

b = $p > 0.05$ versus control (0 week) by student's t test.

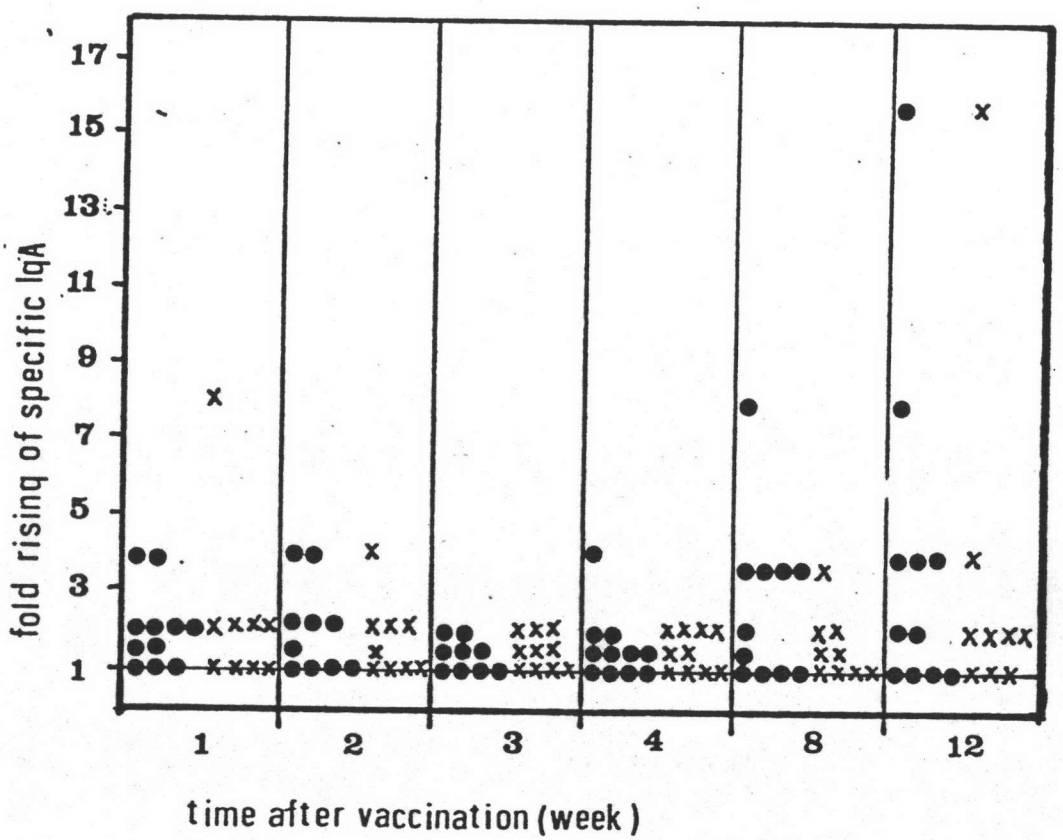

Figure 17 The specific serum IgA anti-CLPS in Vivotif ® and Thai Red Cross groups. Each amount of this antibody is shown as a fold increase over the 1-fold value represent by the solid line, which is equal to the amount of this antibody before vaccination.

● = Vivotif ®, X = Thai Red Cross

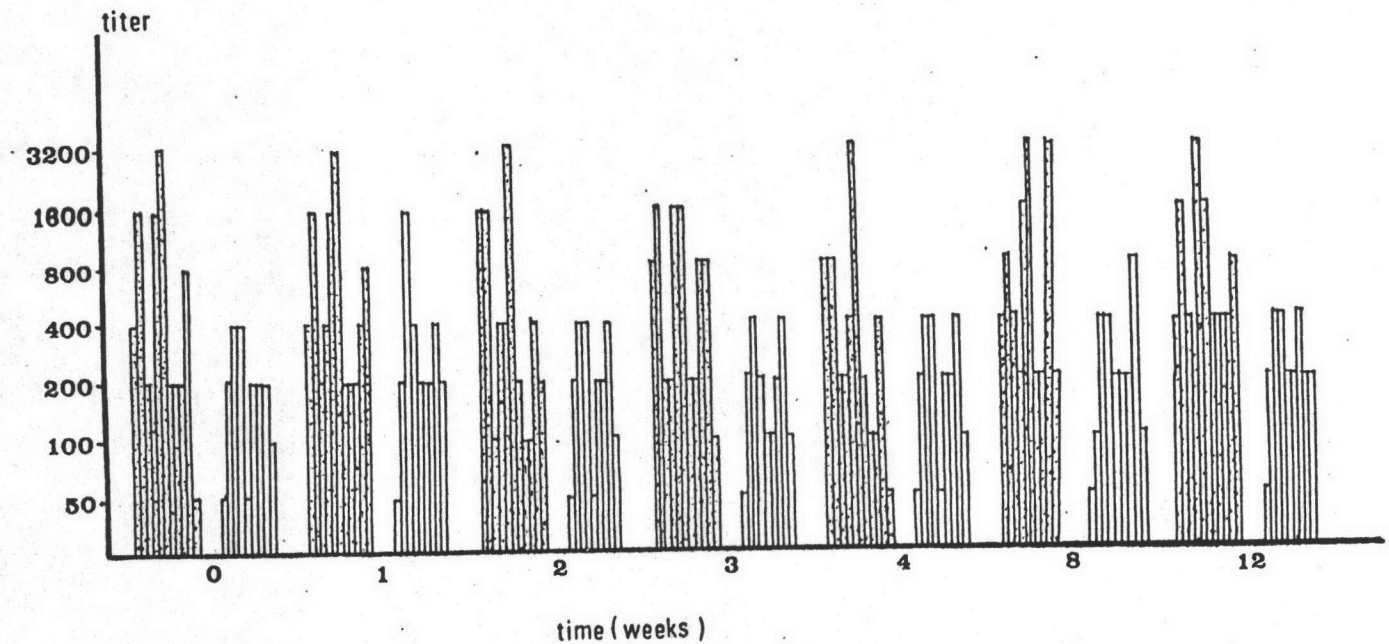

Figure 18 The individual serum IgA of 11 and 9 vaccinees in Vivotif ® and Thai Red Cross groups, respectively.

▨ = Vivotif ®, □ = Thai Red Cross

7. The saliva IgA anti-CLPS response

The IgA response to CLPS antigen in saliva was showed in Table 7 , Figure 19 and Figure 20.

It was found that in Vivotif ® group, saliva IgA after vaccination was not significantly increased ($p > 0.05$) versus the contol (0 week). In the Thai Red Cross group at the 1 st and 2 nd weeks after vaccination, it was significantly increased ($p < 0.05$) versus the control but in the later weeks the levels of saliva IgA decreased to be equal to control.

8. The stool extract IgA anti-CLPS response

The IgA response to CLPS antigen in stool extract was shown in Table 8, Figure 21 and Figure 22.

It was found that in both groups of vaccinated volunteers, the concentration of stool extract IgA after vaccination did not significantly increased ($p > 0.05$) compared to the control (0 week).

9. The intestinal lavage IgA anti-CLPS response

After vaccination, the intestinal lavage IgA were significantly increased compared to the control(0 week) in both groups of vaccinees. In the Vivotif ® group, the

Table 7 The saliva IgA anti-CLPS[a] in vaccinees who received two different typhoid vaccines.

Time	Vivotif ®	Thai Red Cross
Ø week	18.77 ± 7.8	68.58 ± 19.3
1 week	27.4 ± 12.7[c]	108.89 ± 29.Ø[b]
2 weeks	17.63 ± 6.8[c]	93.32 ± 15.Ø[b]
3 weeks	18.77 ± 8.4[c]	86.4 ± 3Ø[c]
4 weeks	2Ø.ØØ ± 17.5[c]	68.57 ± 32.Ø[c]
8 weeks	17.63 ± 6.6[c]	5Ø.39 ± 18.33[c]
12 weeks	18.77 ± 13.4[c]	137 ± 46.8[b]

a = Geometric mean titer ± S.E. of 11 and 9 vaccinees in Vivotif ® and Thai Red cross groups, respectively.

b = $p < Ø.Ø5$ versus control (Ø week) by student's t test.

c = $p > Ø.Ø5$ versus control (Ø week) by student's t test.

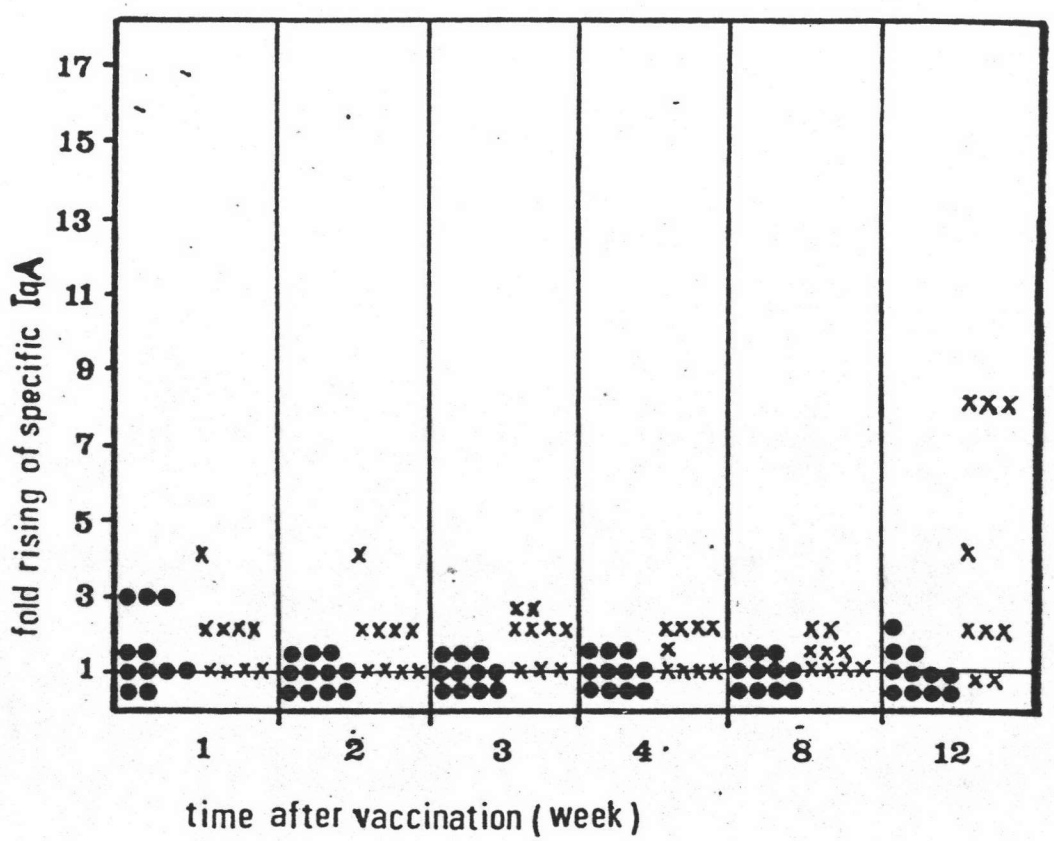

Figure 19 The specific saliva IgA anti-CLPS in Vivotif ® and Thai Red Cross groups. Each amount of this antibody is shown as fold increase over the 1-fold value represent by the solid line, which is equal to the amount of this antibody before vaccination.

● = Vivotif ® , X = Thai Red Cross

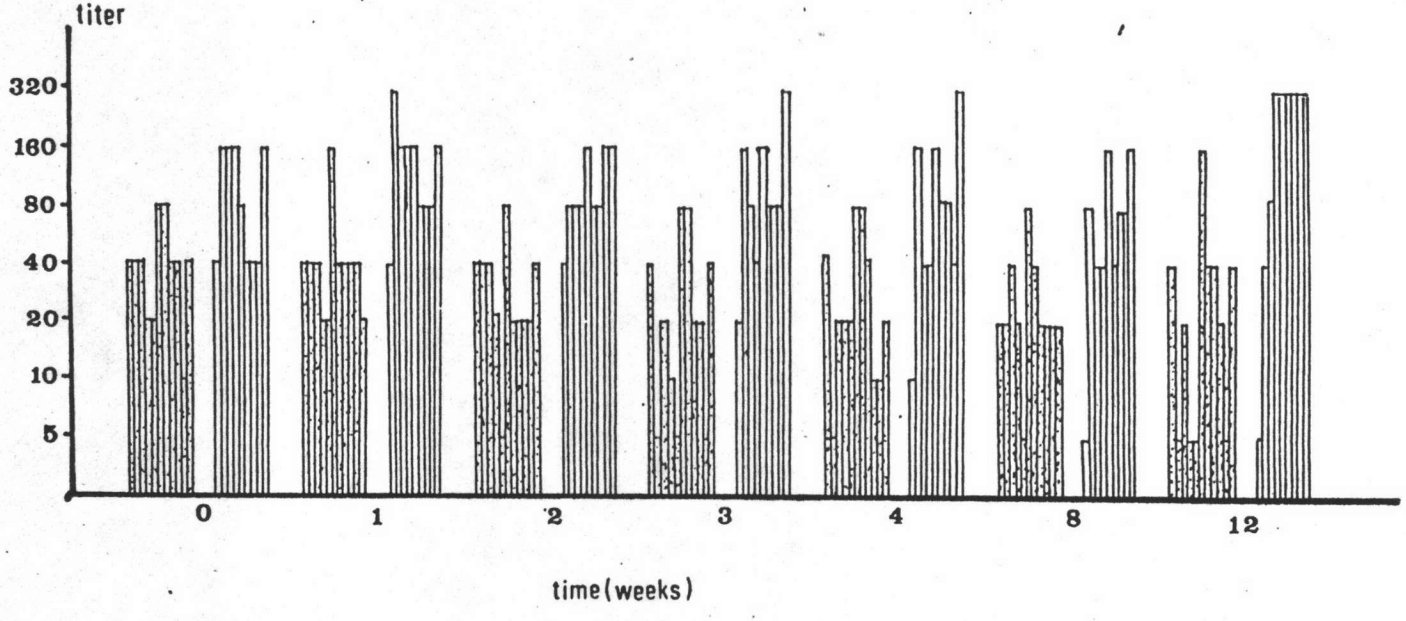

Figure 20 The individual saliva IgA of 11 and 9 vaccinees in Vivotif ® and Thai Red Cross groups, respectively.

▨ = Vivotif ® , □ = Thai Red Cross

Table 8 The Stool Extract IgA anti-CLPS[a] in vaccinees who received two different typhoid vaccines.

Time	Vivotif ®	Thai Red Cross
0 week	175 ± 62	434 ± 441
1 week	200 ± 73[c]	432 ± 421[c]
2 weeks	174 ± 12[c]	434 ± 332[c]
3 weeks	152 ± 62[c]	466 ± 427[c]
4 weeks	248 ± 59[b]	432 ± 320[c]
8 weeks	135 ± 36[c]	432 ± 160[c]
12 weeks	174 ± 73[c]	342 ±161[c]

a = Geometric mean titer ± S.E. of 5 and 9 vaccinees in Vivotif ® and Thai Red Cross groups, respectively.

b = $p < 0.05$ versus control (0 week) by student's t test.

c = $p > 0.05$ versus control (0 week) by student's t test.

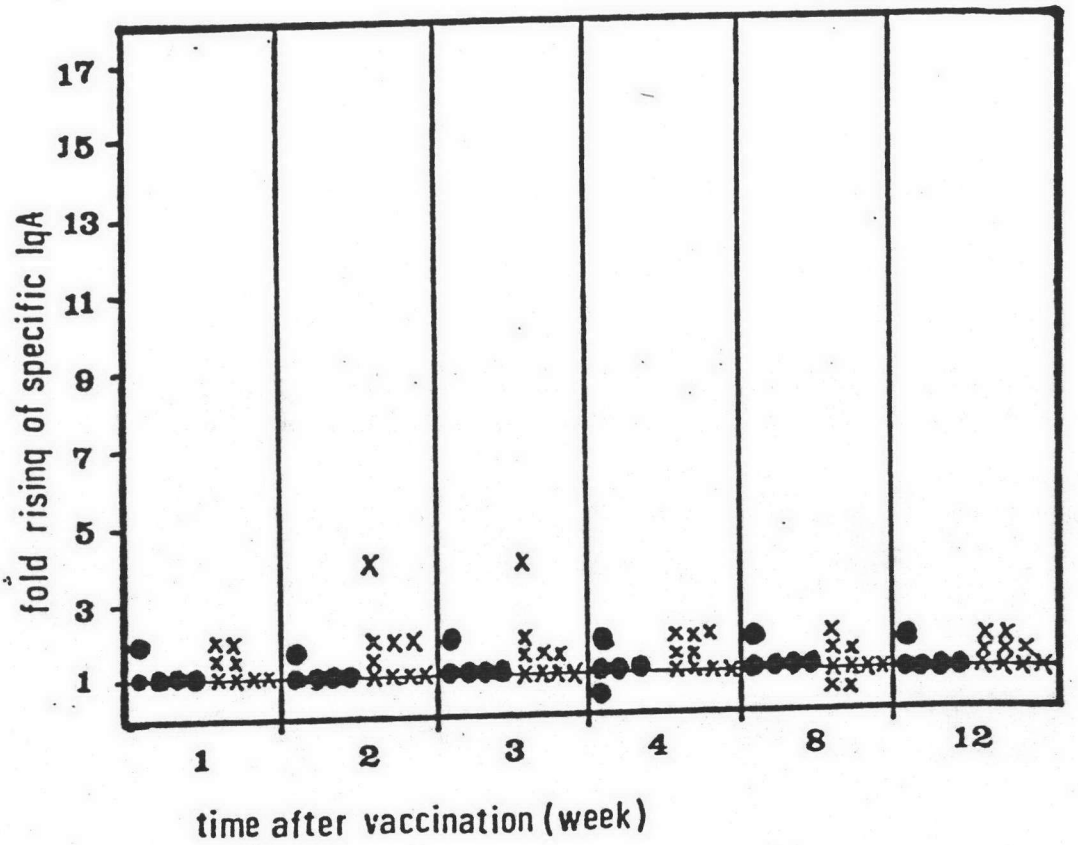

Figure 21 The specific stool extract IgA anti-CLPS in Vivotif ® and Thai Red Cross groups. Each amount of this antibody is shown as a fold increase over the 1-fold value represent by the solid line, which is equal to the amount of this antibody before vaccination.

● = Vivotif ®, X = Thai Red Cross

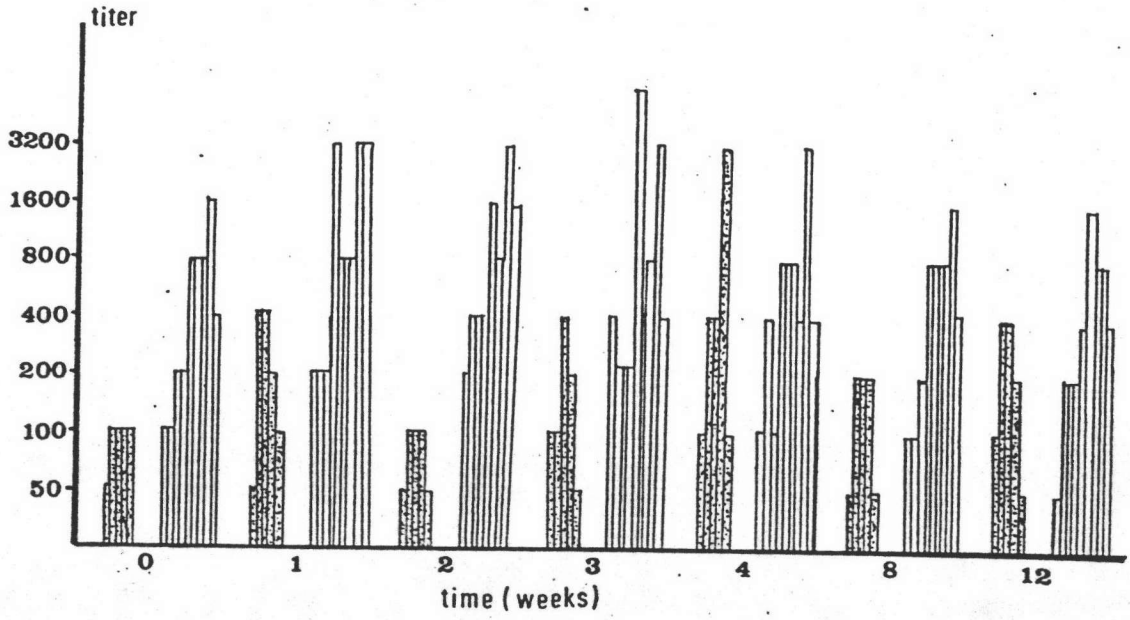

Figure 22 The individual stool extract IgA of 5 and 9 vaccinees in Vivotif ® and Thai Red Cross groups, respectively.

▨ = Vivotif ® , □ = Thai Red Cross

first increase was seen at the 2 nd week after vaccination and the greatest amount of IgA was seen at the 4 th week after vaccination. In the Thai Red Cross group, the first increase of IgA was found at the 1 st week after vaccination and the greatest amount of IgA was seen at the 3 rd week after vaccination at shows in Table 9, Table 10, Figure 23 and Figure 24.

10. A comparison of urease and Alkaline phosphatase as coupled enzymes in the ELISA

Compared the activity (sensitivity and specificity) of the coupled enzymes urease and alkaline phosphatase. The urease conjugate was readily detected as a visual end point; the alkaline phosphatase substrate was detected by reading the O.D. at 405 nm, the titer was determined for these specimens at the 0.4 O.D. level (the substrate control or the background value $\pm$ 2SD).

As illustrated in Table 11 , we found that the titer of ten sera samples from our volunteers measured by the urease conjugate are lower than the titer obtained with alkaline phosphatase conjugate by one or two titer dilutions.

Table 12 , describes the specificity and sensitivity of enzyme urease when compared with enzyme alkaline phosphatase conjugate. It was found that the sensitivity of urease was 77 % and specificity was 100 % .

Table 9 The Local intestinal lavage IgA anti-CLPS[a] in vaccinees who received two different typhoid vaccines.

Time	Vivotif ®	Thai Red Cross
0 week	17.5 ± 28	40 ± 37
1 week	34 ± 30[d]	75 ± 27[c]
2 weeks	53 ± 38[c]	72 ± 30[c]
3 weeks	46 ± 29[f]	99 ± 26[c]
4 weeks	77 ± 35[b]	72 ± 33[c]
8 weeks	75 ± 39[c]	60 ± 19[f]
12 weeks	64 ± 33[c]	37 ± 16[d]

a = Geometric mean titer ± S.E. of 6 and 9 vaccinees in Vivotif ® and Thai Red Cross groups, respectively.

b = $p < 0.01$ versus control (0 week) by student's t test.

c = $p < 0.05$ versus control (0 week) by student's t test.

d = $p > 0.05$ versus control (0 week) by student's t test.

e = $p < 0.1$ versus control (0 week) by student's t test.

f = $p < 0.2$ versus control (0 week) by student's t test.

Table 10 The specific IgA anti-CLPS* from various sources in vaccinees who received two different typhoid vaccines before and after vaccination.

Time	Vivotif ®		Thai Red Cross	
SERUM				
before	375 ± 296	p > 0.05	162 ± 50	p > 0.05
after	442 ± 260		184 ± 75	
SALIVA				
before	18.77 ± 7.8	p > 0.05	68.58 ± 19	p > 0.05
after	19.82 ± 10.9		86.4 ± 18.52	
STOOL EXTRACT				
before	175 ± 62	p > 0.05	434 ± 441	p > 0.05
after	177 ± 52		448 ± 304	
INTESTINAL LAVAGE				
before	17.5 ± 28	p < 0.05	40 ± 37	p < 0.05
after	58.16 ± 34		69 ± 25	

a = Geometric mean titer ± S.E. of vaccinees in Vivotif ® and Thai Red Cross groups, respectively.

b = Geometric mean titer of pool specific IgA after vaccination at 1, 2, 3, 4, 8 and 12 weeks ± S.E. of vaccinees in Vivotif ® and Thai Red Cross groups, respectively.

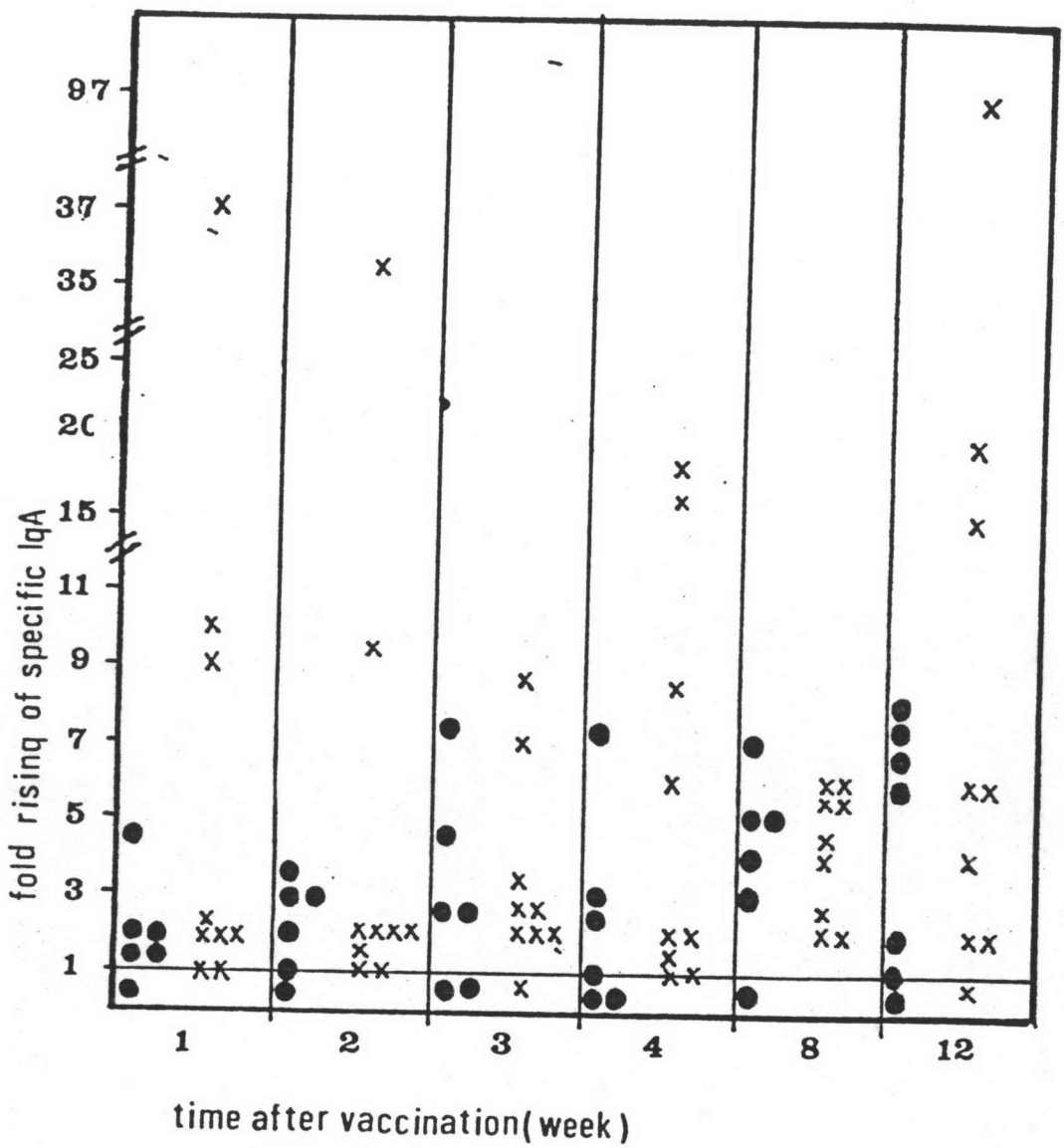

Figure 23 The specific local intestinal lavage IgA anti-CLPS in Vivotif ® and Thai Red Cross groups. Each amount of this antibody is shown as a fold increase over the 1-fold value represent by the solid line, which is equal to the amount of this antibody before vaccination.

● = Vivotif ®, X = Thai Red Cross

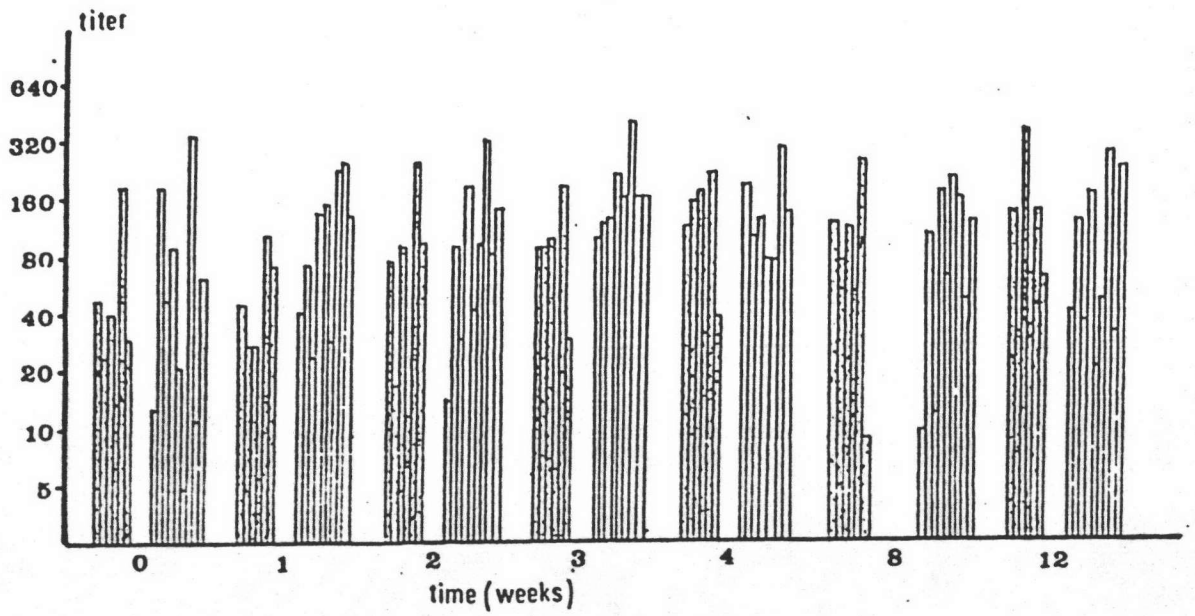

Figure 24 The individual intestinal lavage IgA of 6 and 9 vaccinees in Vivotif ® and Thai Red Cross groups, respectively.

▨ = Vivotif ®, □ = Thai Red Cross

Table 11 Comparison the activity of enzyme urease and alkaline phosphatase conjugates

Serum	1		2		3		4		5		6		7		8		9		10	
dilution	AP[a]	U[b]	AP	U	AP	U	AP	U	AP	U	AP	U	AP	U	AP	U	AP	U	AP	U
1:50	+[c]	+[e]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1:100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
1:200	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-
1:400	+	-[f]	+	-	+	-	+	-	+	-	+	-	+	-	+	-	-	-	-	-
1:800	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
1:1600	-[d]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:3200	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

a = anti-human-α-chain conjugate alkaline phosphatase (dilution 1:500)

b = anti-human-α-chain conjugate urease (dilution 1:100)

c = O.D. at 405 nm > 0.4 within 30 minutes

d = O.D. at 405 nm < 0.4 within 30 minutes

e = Substrate bromocresol purple urea (BCP-urea) change from yellow to complete violet within 30 minutes

f = Substrate BCP-urea color is yellow within 30 minutes

Table 12 Sensitivity and Specificty of Urease compared with alkaline phosphatase

Urease Results	O.D. at 405 nm of PNP [a]	
	> 0.4	< 0.4
Positive [b]	27	0
Negative [c]	8	35

a = substrate for alkaline phosphatase (p-nitrophenyl phosphate)

b = Urea-BCP color change from yellow to violet

c = color did not change (yellow)

$$\text{Sensitivity} = \frac{27 \times 100}{27 + 8} = 77\ \%$$

$$\text{Specificity} = \frac{35 \times 100}{35 + 0} = 100\ \%$$