THE DIAGNOSTIC VALUE OF THE ELISA-Ty TEST FOR THE DETECTION OF TYPHOID FEVER IN CHILDREN

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Abstract. A study on the reliability of an enzyme linked immunosorbent assay (ELISA) for the detection of typhoid fever, the ELISA-Ty test, was conducted, comprising 44 children suffering from bacteriologically confirmed typhoid fever based on the finding of a positive blood culture for *Salmonella typhi*, 44 children with fever caused by diseases other than typhoid fever based on the finding of negative culture of blood, urine and stool for *S. typhi*, and 120 healthy children as controls. This ELISA-Ty test measures the concentration of IgM and of IgG against *S. typhi* in serum. This test is an indirect ELISA test, based on a method that makes use of a mixture of OMPs (outer membrane proteins) in equal proportion serving as antigen, obtained from different strains of *S. typhi* which are prevalent locally, peroxidase goat antihuman IgG or IgM (Sigma) as conjugate and orthophenylenediamine (Sigma) as chromogen of the substrate. The result of the test was obtained through the assessment of the end product, using a micro ELISA reader (Behring) at wave length of 490 nm. The data revealed that the mean absorbent values found in children with typhoid fever, for IgM and IgG, were significantly higher ($p < 0.05$) when compared to those in children with non – typhoid fever as well as to those found in children of the control group. The results of this study confirm that the ELISA-Ty test has a high reliability for the detection of typhoid fever in children, based on the finding of a degree of diagnostic sensitivity as high as 95.45% and 90.91% for respectively IgM and IgG, a diagnostic specificity as high as 93.33% for IgM as well as for IgG, a high diagnostic efficiency (94.32% for IgM and 92.05% for IgG), a high diagnostic positive predictive value of 93.33% for IgM and 93.02% for IgG, high negative predictive values of 95.35% for IgM and 91.11% for IgG for use under clinical as well as under field conditions.

INTRODUCTION

Typhoid fever continues to be an important public health problem in many developing countries, especially in tropical parts of the world (Thong *et al*, 1994). In Indonesia, the disease is still highly endemic, and causes a 3.3% mortality of the total mortality in this country (Bidasari Lubis, 1990). The highest incidence rate of typhoid fever is found in the age group of 3 to 19 years (78%) which consists of school children (Simanjuntak, 1990). The use of vaccination as a strategy to control typhoid fever in Indonesia, has appeared to be disappointing while environmental sanitation can be considered unsatisfactory. Thus, case finding and contact tracing followed by adequate treatment appear to be the key point in the control of typhoid fever in Indonesia. For the purpose of case finding, a reliable, practicable and low cost diagnostic tool is of crucial importance.

A definitive diagnosis of typhoid fever can only be made through the isolation of *S. typhi* in blood or in other specimens (Tsang and Chau, 1992). However the results of blood cultures are still far from satisfactory; the reported incidence rate of positive isolates varies from 48 - 78% (Tsang and Chau, 1992). The relatively low degree of practicability of the blood culture is the other side of the coin. In rural areas where laboratory facilities are very limited, the diagnosis of typhoid fever is mainly based on clinical symptoms of the disease and the four-fold rise in the antibody titers of the Widal test (Bidasari Lubis, 1990; Simanjuntak, 1990; Verdugo-Rodrigues *et al*, 1993; Moehario *et al*, 1995). To date, conventional Widal test is still widely used to support the diagnosis of typhoid fever because it can be very easily carried out and considered a very cheap test. On the other hand this test lacks proper sensitivity as well as specificity (Bidasari Lubis, 1990; Simanjuntak, 1990; Moehario and Sudarmono, 1995).

The use of enzyme-linked immunosorbent assay (ELISA) for the diagnosis of typhoid fever was reported to have a high degree of sensitivity and specificity as well with a moderate degree of practicability (Gam, 1992). The other advantage of the indirect ELISA test is its ability to detect the class of antibody (IgM and IgG) against *S. typhi* (Verdugo-Rodrigues *et al*, 1993; Listyani 1994; Gam, 1992). In children, due to their infrequent subclinical contact with *S. typhi* when compared with adults in the same endemic area, the cut off

value of the ELISA test for typhoid fever may be significantly lower than that in the adults. The above mentioned problems open the way for a farther study with the aim of evaluating the diagnostic value of the micro ELISA test by using OMPs as the antigen (ELISA-Ty) for the detection of typhoid fever in children.

MATERIALS AND METHODS

This study was based on the examination of sera obtained from 208 children divided into 3 groups:

1. A group of 44 children with positive culture (blood) for *S. typhi* and clinical signs and symptoms suggestive of typhoid fever.
2. A group of 44 children with negative culture (blood, urine, and stool) for *S. typhi* but with fever caused by other diseases than typhoid fever.
3. A group of 120 healthy children as controls.

The population under study were children (male and female) of the age group of 0 - 12 years who had not been vaccinated with typhoid vaccine during the previous two years. All patients were not under treatment with corticosteroid or other immunosuppressive drugs during the previous month, did not suffer from diseases that could interfere with the development of the humoral immune response, and did not suffer from malnutrition. The list of nontyphoid diseases with fever comprised dengue, morbili, urinary-tract infections, mumps, malaria, varicella, diphtheria and bronchopneumonia.

Sera obtained from the population under study were tested by using indirect ELISA technique (ELISA-Ty) which is in brief performed as follows. The antigen used in this test was a mixture of equal quantity of outer membrane proteins (OMPs) obtained from 5 different strains of *S. typhi* that are prevalent locally. The OMPs were prepared according to the procedure described previously (Gam, 1992; Verdugo-Rodrigues *et al*, 1993). The optimal concentration of OMPs antigen appeared to be 2.5 μg/ml for IgM as well as for IgG, and the optimal dilution of the serum appeared to be 1 : 500 for IgM and 1 : 1,000 for IgG ELISA-Ty test based on the results of standardization of conditions of each step in the ELISA-Ty test through the use of chequerboard titration. The optimal dilution of conjugate was 1 : 500 for IgM as well as for IgG. One hundred and fifty μl of OMP antigen (2.5 μg/ml) in carbonate buffer solution (pH 9.6) was used to coat the flat bottom wells of the microtiter plate, overnight at 4ºC.

The plate was then washed 3 times with phosphate buffer saline tween 20 (PBS-T) solution with a pH of 7.2 and inverted on dry, absorbent paper to drain the last drops of the wash buffer solution. The plate was afterwards blocked by using 200 μl bovine serum albumin (BSA) 2% (w/v) in 10 mM PBS-T (pH 7.2), incubated for one hour at room temperature and then washed 3 times as mentioned above. During the next step of ELISA-Ty procedure, 150 μl of appropriately diluted human sera (1 : 500 for IgM and 1 : 1,000 for IgG) was dispensed into each well and incubated at 37ºC for one hour.

After 4 washes with PBS-T, 150 μl of a 1 : 500 dilution of peroxidase conjugate goat antihuman IgM (Sigma, catalog No. A-8650) or IgG (Sigma, catalog No. A-8792) was added to each well and incubated at 37ºC for one hour. The plate was washed 5 times with PBS-T followed by the addition of a chromogenic substrate (orthophenylenediamine) (Sigma, catalog No. P-8287) in substrate buffer (containing H_2O_2 0.04%) and afterwards incubated at room temperature for 15 minutes. Finally, 50 μl of stopping solution (H_2SO_4 12.5%) was added to each well to stop the enzymatic reaction. Color development was read by using micro ELISA reader (Behring) at 490 nm (Gam, 1992; Verdugo-Rodrigues *et al*, 1993). The cut off value of the ELISA-Ty test found in 120 normal children in this study was 0.554 absorbent unit for IgM and 0.639 absorbent unit for IgG. The diagnostic value of ELISA-Ty test was assessed based on the determination of the diagnostic sensitivity, the diagnostic specificity, the diagnostic efficiency, the diagnostic positive predictive value and the negative predictive value.

Blood culture in bile - broth media, urine culture and stool culture were carried out according to the standard procedures of the Microbiology Division, Department of Clinical Pathology, Dr Soetomo Hospital in Surabaya Indonesia. A positive result of blood culture served as the gold standard for the confirmation of typhoid fever in this study.

Statistical analysis used in this study was based on the McNemar test and the Student's *t*-test with confidence limit of 0.05 (Conover, 1971).

RESULT

Table 1
The mean absorbent value of IgM and IgG ELISA-Ty tests in sera of children with typhoid fever and children with nontyphoid fever.

Class of antibody	Typhoid fever		Non-typhoid fever		t	p
	Mean	SD	Mean	SD		
IgM	0.997	0.439	0.395	0.131	8.72	< 0.05
IgG	1.111	0.572	0.454	0.176	7.27	< 0.05

Table 2
The mean absorbent value of IgM and IgG ELISA-Ty tests in sera of children with typhoid fever and of healthy children.

Class of antibody	Typhoid fever		Normal control		t	p
	Mean	SD	Mean	SD		
IgM	0.997	0.439	0.391	0.102	9.07	< 0.05
IgG	1.111	0.572	0.454	0.152	7.92	< 0.05

Table 3
The results of IgM and IgG ELISA-Ty tests in sera of children with typhoid and of children with nontyphoid fever.

Type of disease	N_A	Results of the ELISA-Ty tests for IgM				for IgG			
		Positive		Negative		Positive		Negative	
		N_B	%	N_C	%	N_B	%	N_C	%
Typhoid	44	42	95.45	2	4.55	40	90.91	4	9.09
Nontyphoid	44	3	6.82	41	93.18	3	6.82	41	93.18

N_A = number of children examined; N_B = number of positive results; N_C = number of negative results.

Tables 1 and 2 show the results of IgM and IgG ELISA-Ty tests in the group of children with typhoid fever, in the group of children with nontyphoid fever and in the group of healthy children. The mean absorbent values in the group of children with typhoid fever were 0.997 (SD = 0.439) and 1.111 (SD = 0.572) for respectively IgM and IgG. In the group of children with non-typhoid fever the mean absorbent values were respectively 0.395 (SD = 0.131) and 0.454 (SD = 0.176) for IgM and IgG. The difference in the mean absorbent value between the two groups of children was statistically significant ($p < 0.05$) for IgM as well as for IgG (Table 1).

It can be seen in the Table 2 that the mean absorbent values for the IgM and IgG were respectively 0.391 (SD = 0.102) and 0.454 (SD ± 0.152) in healthy children (normal control). A statistical significant difference was found between the group of children with typhoid fever and the group of healthy children in the mean absorbent value for IgM ($p < 0.05$) as well as for IgG ($p < 0.05$) (Table 2).

It can be seen in Table 3, that of the 44 children with typhoid fever, 42 children (95.45%) had positive IgM ELISA-Ty test and 40 children (90.19%) had positive IgG ELISA-Ty test. The diagnostic sensitivity of the ELISA-Ty test in these children was 95.45% for IgM and 90.91% for IgG. In the group of children with nontyphoid fever, 41 of the 44 children (93.18%) showed negative ELISA-

Ty test for both IgM as well as for IgG. The diagnostic specificity of the ELISA-Ty test in these children was 93.18% for both IgM and for IgG. The diagnostic efficiency of the ELISA-Ty test in the study reported here was thus 94.32% for IgM and 92.05% for IgG for the diagnosis of typhoid fever.

The diagnostic positive value of the ELISA-Ty test in this study was 93.33% for IgM and 93.02% for IgG. Besides, its negative predictive value was 93.35% for IgM and 91.11% for IgG for the diagnosis of typhoid fever. Though the diagnostic sensitivity of ELISA-Ty test for IgM was higher compared with that for IgG, the difference was however statistically not significant ($p > 0.05$). Besides, their diagnostic specificity showed no difference ($p > 0.05$).

DISCUSSION

The results of the study reported here indicate that the specific antibody levels against the OMPs antigen of *S. typhi*, with regard to IgM and to IgG in children with typhoid fever, were significantly higher ($p < 0.05$) when compared to those found in children with nontyphoid fever as well as to those found in healthy children. The mean absorbent values of the specific antibodies in children with typhoid fever were 2.5 times higher than those in healthy children. Verdugo-Rodrigues (1993) has found almost the same figure with regard to the ratio of specific antibody levels, *ie* 2.5 - 2.8 times higher in adult patients when compared to those levels found in healthy individuals. The sensitivity for diagnostic purpose of the ELISA-Ty test when used in children can thus be classified as being very high for IgM and as being high for IgG when based on the classification reported by Handojo (1988). Diagnostic sensitivity was found to be 97.73% when results of the ELISA-Ty test for IgM and for IgG in children with typhoid fever were evaluated as being one test. The OMPs antigen is obviously superior to the lipopolysaccharide (LPS) antigen. The rationale behind this finding must be based on the fact that the OMPs antigen (especially the porins) is more immunogenic than the LPS antigen. The OMPs antigen has a greater potency to stimulate the B-cell for the production and release of high concentrations of immunoglobin. This phenomenon is mainly based on the fact that OMPs antigen is more stable to the effect of proteolysis brought about by proteolytic enzymes. A stable state of the OMPs antigen may therefore be maintained in blood for a longer period of time.

It is worthy to note farther that OMPs antigen has the capacity to enhance cell mediated immunity, thereby inducing long lasting immune responses against the *S. typhi* (Gam, 1992; Tandra and Soewandojo, 1986). Based on data obtained from adult patients in areas where typhoid fever is endemic, Handojo and Listyani (1995) reported that sensitivity for diagnostic purpose of the ELISA test for IgG was significantly higher ($p < 0.05$) than that for IgM.

However, in the study reported here, sensitivity for diagnostic purpose of the ELISA-Ty test for IgM did not differ significantly ($p > 0.05$) from that for IgG, while the children under study did also come from the same endemic area. It is enticing to speculate that the above findings must be based on the possibility that most of the children under study were less prone to be exposed to a low dose of infection with *S. typhi*, not enough for the development of the disease (subclinical infection). In case these children under study got ill following infection with a high dose of bacilli, the immune response generated will have the pattern of a primary infection, which means the level of IgM antibody is higher than that of IgG.

The situation will be different when dealing with adults in endemic area. Most of them are more prone to be exposed to a low (subclinical) dose of infection with *S. typhi*, thereby resulting in the generation of immune response, which is inherent to the pattern of a secondary immune response (secondary infection) if they got ill. This pattern of immune response is characterized by the emergence of a higher concentration of IgG.

The fact, that the children under study still had positive blood culture of *S. typhi*, explains that they were still in the early stage of their disease (the first week); IgM and IgG antibody can be expected to be present in equal and low concentration (Hischl *et al*, 1985). That is why no significant difference was found in the diagnostic sensitivity between IgG and IgM. As was reported earlier, no significant difference was found in the diagnostic specificity between the IgM ELISA-Ty test and the IgG ELISA-Ty test. This may be accounted for by the fact that both tests have used the same antigen. The other materials used in these tests were also almost similar and were produced by the same factory (Sigma). It can be expected therefore that the specificity profile of the mate-

rials used in both test are almost similar. No cross-reaction was encountered between the OMPs antigen of *S. typhi* and the antigen of *Escherichia coli* found in patients under study suffering from a concomitant urinary tract infection.

Due to its high diagnostic specificity of this OMPs antigen, when used in the ELISA-Ty test, the administration of the mentioned test has to be contemplated moreover in areas where typhoid fever is endemic and where similar strains of *S. typhi* are prevalent.

The use of a mixture (in equal proportions) of 5 locally prevalent strains of *S. typhi* to produce OMPs antigen for the ELISA-Ty test in this study was thought to be the main reason responsible for the high diagnostic sensitivity and specificity of this test. The mixture of the OMPs antigens derived from 5 locally prevalent strains of *S. typhi* gives rise to a broad spectrum antigen for the ELISA-Ty test resulting in the very high degree of diagnostic sensitivity of this test, while the locally prevalent strains of *S. typhi* used in this study are presumed to be responsible for the high degree of specificity of the OMPs antigen for the ELISA-Ty test. It is important to note that the Widal test, which makes use of antigen that is obtained from strains of *S. typhi* that are prevalent locally, has a significantly higher diagnostic sensitivity and specificity when compared to other Widal tests that use antigen which are obtained from imported strains or other strains of *S. typhi* that are not locally prevalent. Evidence on this finding was reported by Suwahyo (1979) and by Setyawati (1997).

Reproducibility of the ELISA-Ty test is another point to be given due consideration. The reproducibility of the ELISA-Ty test in this study could be considered as very good.

The within - run coefficient of variation (CV) was found to be 6.42% for IgM and 5.76% for IgG whereas the between days CV appeared to be 10.41% for IgM and 12.66% for IgG. Both the mentioned CV-s were still within the limits permitted for the ELISA test (Schuurs and Van Weeman, 1977).

From practical point of view, the ELISA-Ty test is a moderate practical test. The use of micro ELISA reader is however still indispensable for the reading of the result of the test. The low cost of the ELISA-Ty test is another advantage, which is less than US$ 1. Analysis of data obtained during the performance of the study reported here indicates that the ELISA-Ty test is an eligible and cheap diagnostic tool for the detection of typhoid fever in children. This test has however a moderate degree of practicability.

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