DIAGNOSTIC VALUE OF DOT-ENZYME-IMMUNOASSAY TEST TO DETECT OUTER MEMBRANE PROTEIN ANTIGEN IN SERA OF PATIENTS WITH TYPHOID FEVER

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Abstract. Typhoid fever is still an important public health problem in many developing countries especially in tropical parts of the world, as in Indonesia. This problem opens the way for a further study with the aim of finding an alternative serological test with a high degree of reliability for the detection of typhoid fever. Given the above mentioned purpose, a study on the reliability of a laboratory test, the dot-enzyme-immunoassay outer membrane protein (DOT-EIA-OMP) was conducted comprising sera from 207 subjects (44 adult typhoid patients, 43 adult nontyphoid patients and sera from 120 adult healthy individuals serving as controls.

The result of the study revealed that the diagnostic sensitivity of the DOT-EIA-OMP test for the detection of typhoid fever can be classified as high (93.16%), the specificity as moderate (76.74%), the efficiency (accuracy), positive predictive value and negative predictive value as high (85.06%, 80.39% and 91.66% respectively). The within run and between days reproducibility of this test was very high (CV=0%). Analysis of data obtained indicated that the DOT-EIA-OMP test was a reliable screening test for the establishment of the diagnosis of typhoid fever in health centers with simple laboratory facilities. The application of this test has to be more contemplated in countries where the cost of laboratory test is a problem.

INTRODUCTION

Typhoid fever, a febrile illness with an annual prevalence of 16 million cases throughout the world and a case fatality rate of 3.5-5% in developing countries, is a serious health problem affecting many developing countries, including Indonesia (Punjabi, 1995; Qadri *et al*, 1990; Sukosol *et al*, 1995). It is highly endemic in Indonesia and most of the patients are young; the national case fatality rate of hospitalized patients was between 2-3.5% (Qadri *et al*, 1990; Sukosol *et al*, 1995). Growing urbanization resulting in crowded living space, bad environmental sanitation, water supplies unfit for drinking, little knowledge on the pathogenesis and pathogenecity of the causative agent, difficulty related to the establishment of a definite diagnosis and the prevalence of drug resistant bacteria, are some of the factors that may have affected the endemicity of the disease (Qadri *et al*, 1990; Sukosol *et al*, 1995).

A definite diagnosis of typhoid fever is obtained by monitoring clinical manifestations, performing serologic tests and detecting *Salmonella typhi* in blood or bone marrow. The often indistinct clinical manifestations of typhoid fever make it difficult for clinicians to confirm the diagnosis. The Widal test that may support the diagnosis is not sensitive enough and not highly specific, especially in endemic areas such as Indonesia. Cultivation of bacilli from blood and other body fluids is time consuming, and needs to be performed using adequate instruments and skilled laboratory technicians, yet its sensitivity is low, *ie* about 40% (Baron, 1990; Punjabi *et al*, 1995).

Given the above situation, every effort has been made to find a test that is confirmative and that enables the detection of the disease at its early stage. The dot-enzyme-immunoassay (DOT-EIA) used to detect outer membrane protein (OMP) antigen in sera of patients with typhoid fever may serve as an alternative, as this test is practical, inexpensive, highly sen-

sitive, specific and applicable even in primary health centers.

MATERIALS AND METHOD

This laboratory study was performed on sera obtained from 207 subjects divided into 3 groups:

1. A group of 44 adults patients (24 males and 20 females aged 15-60 years) with positive blood culture for *S. typhi* and / or with positive polymerase chain reaction (PCR) for *S. typhi*, and / or with a four fold increase of the titer of Widal test, and with clinical signs and symptoms suggestive of typhoid fever.

2. A group of 43 adult patients (22 males and 21 females aged 15-42 years) with negative blood, urine and stool culture for *S. typhi*, with negative PCR test for *S. typhi* and with negative Widal test, but with fever caused by other diseases than typhoid fever.

3. A group of 120 healthy adults as control.

In the list of nontyphoid diseases with fever were dengue, malaria, hepatitis, pneumonia, urinary-tract infections, paratyphoid fever A and B.

Sera obtained were tested by dot-enzyme-immunoassay to detect OMP antigen (DOT-EIA-OMP). The primary antibody used to detect the OMP antigen in sera was anti-OMP polyclonal antibody (rabbit IgG against OMP) obtained from a rabbit (body weight 2.5 kg) which had received an intramuscularly injection of 0.25 μg/μl of a mixture of equal quantities of OMPs derived from 5 different strains of *S. typhi* that are locally prevalent.

The OMPs were prepared according to the procedure as described previously (Gam, 1992; Verdugo-Rodrigues *et al*, 1993 a,b). In order to obtain anti-OMP polyclonal antibody, rabbit hyperimmune sera were purified by affinity chromatography technique using Hi Trap column (protein-A-sepharose column) (Hebert, 1996; Pang *et al*, 1991).

The conjugate used in this test was alkaline phosphatase conjugate goat antirabbit IgG (Sigma) and the chromogenic substrate used was 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium (Promega) in Tris buffer solution containing 0.1 M NaCl and 50 mM $MgCl_2$.

DOT-EIA-OMP test procedure

As results of chequerboard titration (Leung *et al*, 1991; Portsmann and Kiessig, 1992; Kemeny, 1992), the optimal serum dilution was 1:8, the optimal anti-OMP polyclonal antibody dilution was 1:150, the optimal conjugate dilution was 1:1,500 and the optimal reading time was 5 minutes.

The DOT-EIA-OMP test is a modification of the method used by Sadallah *et al* (1990) and is in short performed as follows. One μl appropriately diluted human sera (1:8) was added to 5x10 mm nitrocellulose paper with a pore size of 0.45 μm (Optitran) and left to dry at room temperature. The nitrocellulose paper was afterwards blocked with 5% skimmed milk in buffer blocking solution. After one hour incubation at room temperature the nitrocellulose paper was washed 3 times with phosphate buffer saline tween 20 (PBS-T) solution with a pH of 7.4 during a 10 minutes washing period each time. During the next step, the nitrocellulose paper was soaked in the same chamber containing a 1:150 dilution of anti-OMP polyclonal antibody in PBS solution with pH of 7.2 and incubated for one hour at room temperature on an oscillator.

After 3 washes with PBS-T solution as mentioned above, the nitrocellulose paper was soaked in the same chamber containing a 1:1,500 dilution of alkaline phosphate conjugate goat antirabbit IgG in PBS solution (pH 7.4) containing 0.2% gelatin and 0.2% bovine serum albumin (BSA) and incubated for one hour at room temperature. The nitrocellulose paper was washed again with PBS-T solution, three times as mentioned above followed by the addition of a chromogenic substrate (10 μl NBT and 5 μl BCIP) in 10 ml substrate buffer solution containing 0.1M NaCl and 50 mM

$MgCl_2$ with pH of 9.5. Incubation was made afterwards in a dark room for 5 minutes and then washed with distilled water to stop the reaction and the result of the test was made discernible with naked eyes.

The result was positive if the intensity of the blue dot was the same or sharper when compared to the positive control obtained by making a 1:32 dilution of 0.25 μg/μl OMP in PBS solution (pH 7.2) (Chaicumpa *et al*, 1992; Handojo, 1996; Leung, 1991).

Blood, urine and stool cultures and the PCR test for *S. typhi* (Prihatini, 1996) were done according to the standard procedures of the Microbiology Division, Department of Clinical Pathology, Dr Sutomo Hospital in Surabaya (Baron, 1990; Cheesbrough, 1985). The Widal tube test was performed by using antigen obtained from locally prevalent strains (5 strains) of *S. typhi*. The cut-off value of the Widal test was based on the titer of 1:200 (Prihatini *et al*, 1982).

RESULT

The first stage of the study was to find the optimal serum dilution for the DOT-EIA-OMP test. The results of the study revealed that of the 120 normal sera tested with the DOT-EIA-OMP test, 17 sera (97.5%) showed negative results at a serum dilution of 1:8. Given the above data, examination of other samples in this study was based on a 1:8 serum dilution in PBS solution (pH 7.2).

The second stage of the study covered 44 patients with typhoid fever and 43 patients with nontyphoid fever, and made use of the assessment of the diagnostic value (diagnostic sensitivity, specificity, efficiency, positive, and negative predictive value) of the DOT-EIA-OMP test. The results were summarized in Table 1 and Table 2

It can be seen in Table 1 that of the 44 patients, 26 (59%) showed positive blood culture and PCR test, but negative Widal test, 11 patients (25%) showed negative blood culture but positive PCR as well as Widal test, and 7 patients (16%) showed only positive PCR test. Out of the 44 patients considered to have typhoid fever, 41 patients (93.16%) had positive DOT-EIA-OMP test. The diagnostic sensitivity of the DOT-EIA-OMP test was thus 93.16%.

Table 2 shows that 33 patients (76.74%) in the group of 43 nontyphoid patients had negative DOT-EIA-OMP test. The diagnostic specificity was thus 76.74%.

Table 1
Results of DOT-EIA-OMP test in typhoid group.

No.	Typhoid fever patient criteria	DOT-EIA-OMP test				
		Positive		Negative		Total
		No.	%	No.	%	No.
1	Positive blood culture Positive PCR Negative Widal test	26	100	0	0	26
2	Negative blood culture Positive PCR Positive Widal test	10	90.90	1	2.28	11
3	Negative blood culture Positive PCR Negative Widal test	5	71.42	2	4.64	7
Total		41	93.16	3	6.84	44

Table 2
Results of the non-typhoid fever group.

Criteria of non-typhoid patients	DOT-EIA-OMP test				
	Positive		Negative		Total
	No.	%	No.	%	No.
Acute hepatitis	0	0	6	100	6
Malaria	0	0	7	100	7
Pneumonia	0	0	2	100	2
Urinary tract infection	0	0	3	100	3
Dengue hemorrhagic	8	34.78	15	65.21	3
Paratyphoid A	1	100	0	0	1
Paratyphoid B	1	100	0	0	1
Total	10	33.26	33	76.74	43

Two of the 10 nontyphoid patients who produced a false positive result of DOT-EIA-OMP test, appeared to have paratyphoid fever (A and B).The other 8 patients had dengue hemorrhagic fever. The diagnostic efficiency (accuracy) of the DOT-EIA-OMP test in the study was thus 85.06%. The diagnostic positive predictive value of the DOT-EIA-OMP test was 80.39% and the negative predictive value was 91.66%.

DISCUSSION

The aim of the study was to find a laboratory test with a high degree of sensitivity which may serve as an alternative to replace other tests so far available for the direct and indirect detection of *S. typhi* in specimens that have to be tested. Besides its diagnostic value, the test has to be practical and a low cost test, especially when applied in developing countries (Handojo, personal communication). The eligibility of a laboratory test was thus assessed on the basis of it being a reliable test, easy to carry out, and inexpensive.

The diagnostic sensitivity of a laboratory test has to be assessed on the basis of its eligibility to produce a number of positive results as high as possible for the establishment of the diagnosis. The antigen in specimens tested by DOT-EIA-OMP was the outer membrane protein (OMP) which was indicative for the presence of *S. typhi*. This DOT-EIA-OMP with a diagnostic sensitivity of 93.16%, can be considered as high when based on the classification made by Handojo (1988). This has been made possible by the use of nitrocellulose paper which is a solid phase for the EIA test with a high potency of binding antigens even when present in a very low concentration (Porstmann, 1992).

It was interesting to note that of the 26 patients considered to have typhoid fever based on a positive blood culture, and a positive PCR test, all (100%) produced a positive result by the DOT-EIA-OMP test. The above finding indicates that for the achievement of a positive result with blood culture a great number of bacilli is needed. Of the 11 patients suffering from typhoid fever based on the positive PCR test, a positive Widal test but a negative blood culture, 10 patients (90.9%) had positive results of examination with the DOT-EIA-OMP test which means that even a small number of OMP antigen present in specimens may suffice for the achievement of a positive result with the DOT-EIA-OMP test. However, when diagnosis of typhoid fever was based on only a positive PCR test, of the 7 patients considered to have typhoid fever, 5 patients (76,42%) had positive results with the DOT-EIA-OMP test.

Basing on the above finding, when only

a very small number of bacilli is present in tested specimens, the PCR test for *S. typhi* has a higher degree of sensitivity when compared with the DOT-EIA-OMP test. The diagnostic specificity of the DOT-EIA-OMP test in this study (76.74%) can be classified as being of moderate degree according to the criterion of Handojo (1988).

False positive results were found in only two groups of patients, namely, the group with paratyphoid fever (2 out of the 2 patients with paratyphoid fever A and B) and the group with dengue hemorrhagic fever (8 out of the 23 patients). No false positive result was found in other groups with nontyphoid fever (hepatitis, malaria, pneumonia, and urinary tract infection).

The incidence of cross reaction with antigen of *S. paratyphi* A and B may be neglected as treatment and management of paratyphoid fever and typhoid fever are not different. However, cross reaction with the antigen of dengue virus may influence the interpretation of the results as dengue fever is often a differential diagnosis of typhoid fever, moreover in areas where both diseases are prevalent.

The polyclonal antibody against the OMPs antigen used as the primary antibody for the DOT-EIA-OMP test may be associated with the high prevalence of false positive results in the two groups of nontyphoid fever patients (paratyphoid and dengue). The alkaline phosphatase conjugate of goat antirabbit IgG has met with the requirement of an adequate test material during quality control done by the manufacturer and did therefore not substantially interfere with the specificity of the test. Different from the blood culture and the Widal test, the DOT-EIA-OMP test is not influenced by any antibiotic treatment given previously.

Basically, the diagnostic accuracy (efficiency) of this test for the detection of typhoid fever was quite high (85.06%). Reproducibility of the DOT-EIA-OMP test is another point to be given due consideration. In this study it can be considered excellent. The within run as well as the between days coefficient of variation (CV) was 0%. From a practical point of view, the DOT-EIA-OMP is a test with eligibility of moderate degree. The use of micropipette is crucial for the test. Unfortunately this equipment is not always at hand in health centers. The low cost of the DOT-EIA-OMP test is an added advantage, which is approximately US$ 1 per test. The moderate degree of specificity of the test may be associated with the use of polyclonal antibody as the primary antibody. This opens the way for further clinical studies on the use of monoclonal instead of polyclonal antibody.

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