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# PLASMA ENDOTOXIN IN TYPHOID FEVER

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## INDEXING WORDS

endotoxemia; diagnosis; infectious diseases; endotoxin-specific limulus test; Indonesia

## SYNOPSIS

Plasma endotoxin contents of the patients with sepsis or typhoid fever were measured by two sophisticated chromogenic limulus tests; Endospecy and Toxicolor tests. Endospecy test is the endotoxin-specific test and Toxicolor is responsible for both endotoxin and (1, 3)-β-D-glucan. Plasma was pretreated by our new PCA

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method which resolved the problem as to the detection of a lesser amount of plasma endotoxin when pretreated by the conventional PCA method. Although Toxicolor values have been reported to exceed more than the Endospecy value, under complicated pathophysiological situations, almost all specimens of these patients had a similar value, except in one expired septic shock case. In 18 typhoid fever cases, *Salmonella typhi* was isolated only from the bile in 5 cases, however endotoxemia occurred in 11 cases (61.1%). Within the first 4 days, the incidence of endotoxemia was higher (10/14, 71.4 %). These results suggest that endotoxin assay seemed to be a useful tool for the diagnosis of typhoid fever.

## INTRODUCTION

Typhoid fever is a major health problem in developing countries, and should not be overlooked when persons return from traveling in endemic areas. Therefore, effective vaccinations and exact diagnoses are needed. [3, 16]

It has been thought that endotoxin plays an important role as to general symptoms of typhoid fever. Endotoxin is a cell wall component of gram-negative bacteria and has many potent biological activities. Especially, endotoxin shock is an irreversible pathological event which sometimes leads to a serious outcome. Two significances exist for measurement of plasma endotoxin in typhoid fever; 1) diagnosis of the disease and 2) to clarify the pathology of endotoxemia.

The endotoxin assay method, limulus test, was developed using horseshoe crab amebocyte lysate, [9] and, based on the elucidation of gel formation reaction of the limulus test, a quantitative chromogenic limulus test was developed (Toxicolor test; Seikagaku Corp., Tokyo, Japan). [10] Furthermore, a sophisticated endotoxin-specific test (Endospecy test, Seikagaku Corp.) has been recently improved. [13]

There exist interfering factors in human plasma for the limulus test and many methods have been proposed as a pretreatment method of plasma. In the chromogenic limulus test kits, a perchloric acid (PCA) method [12] has been presented.

We have resolved the problem as to the detection of a lesser amount of endotoxin in plasmas pretreated with PCA method and we devised the new PCA method. [7)]

In the present study, we attempted to measure plasma endotoxin levels in the plasmas of patients with typhoid fever, using the endotoxin-specific limulus test with the new PCA method and relied on adequate diagnosis of typhoid fever.

## CASES AND METHODS

*Patients and blood sampling*

The subjects were 21 patients (51 specimens); 18 patients suspected with typhoid fever and hospitalized in the Denpasar General Hospital, Indonesia; 2 with meningitis and one encephalitis case. In two meningitis cases, bacterial culturing was not performed. Typhoid fever was suspected by a high fever (more than 38$^0$C), rose spots, relative bradycardia, splenomegaly, and a typhoid tongue upon admission, and diagnosed by the positive bacterial culture of *Salmonella typhi* from the specimens or by a single Widal test at admission. Blood samples for endotoxin assay were collected using heparinized disposable syringes (Terumo, Tokyo, Japan), put into endotoxin-free centrifuging tubes (γ-ray sterilized plastic tube; Falcon, U.S.A), and then centrifuged at 3,000 rpm for 40 sec to obtain platelet rich plasma. The plasmas were stored at -80 $^0$C in sterile plastic tubes (Nunc Cryotube, Roskilde, Denmark) until use.

*Endotoxin assay*

Endotoxin was determined by both chromogenic reagents, Endospecy test (Seikagaku Corp., Tokyo, Japan) and conventional chromogenic test (Toxicolor test, Seikagaku Corp.). In the Endospecy reagent, β-D-glucan-sensitive factor, factor G, is removed from the limulus lysate to be endotoxin-specific. These limulus tests utilize amebocyte lysate obtained from the Japanese horseshoe crab (*Tachypleus tridentatus*). In these reagents, a synthetic chromogenic substrate, n-tert-

butoxycarbonyl-L-leucyl-L-glycyl-L-arginine-p-nitroaniline, was added to the limulus lysate as the substrate for the clotting enzyme, and used for quantitative measurement. At first, plasma was treated by the new PCA method. Briefly, 100 μl of 0.18N NaOH was added to a 100 μl aliquot of test plasma and incubated at 37$^0$C for 5 min. Then, 100 μl of 0.32 M PCA was added, and further incubation was performed at 37 $^0$C for 10 min. The precipitate was dissolved in 200 μl of 0.18N NaOH by vigorous stirring with a vortex mixer: 50 μl of the solution was transferred to another tube and 50 μl of 0.2 M Tris HCI buffer (pH 8.0) was added. A 100 μl aliquot of the Endospecy or Toxicolor reagent was added to 100 μl of treated plasma, mixed for several sec, and then incubated at 37 $^0$C for 30 min. The amount of p-nitroaniline released from the substrate was detected after diazo-coupling by the absorbance of the solution at 545 nm. Endotoxin contents were calculated from a standard curve obtained by standard lipopolysaccharide solution (*Escherichia coli* 0111: B4; Difco, Detroit, MI). The cut-off value of plasma endotoxin was defined as less than 9.8 pg/ml. [7] All glassware was sterilized in a dry-oven at 250 $^0$C for 2 hr. Aluminum caps were used on the test tubes to prevent endotoxin contamination.

## RESULTS

*Plasma endotoxin levels obtained by Endospecy test*

The results of plasma endotoxin assayed by Endospecy in 51 specimens of 21 cases are shown in Table I, and the incidence of endotoxemia is shown in Table II. The incidence of endotoxemia was found to be high in typhoid fever; 11 of 18 cases (61.1%). Plasmas of 14 cases were collected and tested within the first 4 days; 10 cases showed endotoxemia (71.1%). In 5 cases with typhoid fever *S. typhi* was isolated from the bile and endotoxin was positive in 4 cases. *Pseudomonas aeruginosa* was isolated from blood in 4 cases and endotoxemia was associated. In case No. 7, septic shock occurred after bacteremia and

Table I. Plasma endotoxin in infectious diseases.

| Case | Diagnosis | Widal test[1] | treatment[2] | Plasma endotoxin[3] | Culture[4] |
|---|---|---|---|---|---|
| 1 | Purulent meningitis | | | 4.4 (2) / 7.5 (3) | |
| 2 | Purulent meningitis | | | 5.0 (5) / 10.0 (2) | |
| 3 | Typhoid fever | Ty O 1:160 (1) | CP | 45.5 (4) | *P. aeruginosa* (blood, 4) / N.D. (bile, 4 ) |
| 4 | Typhoid fever | Ty H 1:80 (1) | TP | 51.2 (4) / 40.0 (8) 33.5 (11) / 19.0 (16) | *S. typhii* (bile, 2) / *S. aureus* (blood, 4) |
| 5 | Typhoid fever | Ty O 1:160 (1) | CP | 36.5 (4) / 35.3 (8) | *P. aeruginosa* (blood, 4) |
| 6 | Typhoid fever | Ty H 1:320 (6) | Methycole / Cotrimoxazole | 1.0 (6) / 1.0 (7) / 6.6 (12) | N.D. (blood, 7) / N.D. (bile, 14) |
| 7 | Typhoid fever | Ty H 1:80 (1) Ty H 1:160 (10) | ABPC / AMPC / TP | 655.8 (3) / 9.2 (6) | N.D. (bile 1) / *P. aeruginosa* (blood, 2) / Septic shock (13) / Death (17) |
| 8 | Typhoid fever suspected | Ty O <1:20 (1) | CP | 21.3 (1) / 12.4 (4) | *P. aeruginosa* (blood, 1) / N.D. (bile, 3 ) |
| 9 | Typhoid fever | PA H 1:320 (1) | CP | 2.6 (4) | |
| 10 | Typhoid fever | Ty H 1:160 (1) Ty H 1:320 (8) | CP / ABPC | 1.3 (12) | N.D. (bile, 8) |
| 11 | Typhoid fever | PA O 1:320 (1) | Cotrimoxazole | 60.3 (3) | N.D. (bile, 3) |
| 12 | Typhoid fever | Ty O 1:320 (1) | CP | 1.3 (2) / 0.7 (5) | N.D. (bile, 6) |
| 13 | Typhoid fever | Ty H 1:320 (1) | CP | 3.9(3)/ 4.6 (7) | *S. typhi* (bile, 4) |
| 14 | Typhoid fever | PA H 1:320 (1) PA H 1:320 (12) | ABPC | 1.0 (7) / 1.0 (10) / 1.0 (13) / 1.0 (16) / 3.3 (19) | N.D. (bile, 5) |
| 15 | Typhoid fever | Ty H 1:320 (1) | TP | 274.1 (2) / 4.6 (5) / 1.3 (8) | *S. typhi* (bile, 3) |
| 16 | Typhoid fever | Ty O 1:80 (1) | CP | 1.3 (9) / 1.0 (12) | *S. typhi* (bile, 3) |
| 17 | Typhoid fever | Ty H 1:160 (1) | CP | 4.6 (2) / 1.3 (5) / 26.9 (8) / 37.4 (11) / 37.4 (14) | N.D. (bile, 4) |
| 18 | Typhoid fever | PA H 1:320 (1) PA H 1:80 (29) | TP / Contrimoxazole | 3.9 (1) / 373.1 (4) / 23.6 (7) / 695.1 (10) / 55.7 (13) / 315.4 (16) / 611.2 (19) | N.D. (bile, 3) / *Streptobaccilus* (blood, 13) / *Enterbacter* sp. (30) |
| 19 | Typhoid fever | Ty H 1:80 (1) | CP | 32.8 (3) | *S. typhi* (bile, 1) |
| 20 | Typhoid fever | Ty O 1:640 (1) Ty O 1:320 (4) | CP | 19.7 (2) | N.D. (bile, 2) |
| 21 | Encephalitis | Ty O <1:20 | ABPC | 7.2 (1) / 2.6 (4) | N.D. (bile, 3) |

1) Ty O; *S. typhi* O agglutinin, Ty H; *S. typhi* H agglutinin, PA O; *S. paratyphi* A O agglutinin, PA H; *S. paratyphi* A H agglutinin (day of illness),
2) CP; Chloramphenicol, TP; Thiamphenicol, ABPC; Ampicillin, AMPC; Amoxicillin, 3) pg/ml (day of illness), 4) N.D.; not detected (specimen, day of illness)

endotoxemia. In case No. 18, bacteremia and endotoxemia was followed by disseminated intravascular coagulation and the patient expired.

In purulent meningitis cases (Nos. 1 and 2), plasma endotoxin was almost negative except for one specimen which showed slight positive endotoxemia.

Table II. Incidence of endotoxemia in infectious diseases.

| | Positive cases / Total cases |
|---|---|
| Typhoid fever[1)] | 11 / 18 |
| Purulent meningitis | 1 / 2 |
| Encephalitis | 0 / 1 |

[1)] One suspected case was included

*Comparison of the results obtained by Toxicolor and Endospecy tests*

All 51 plasma samples were assayed by both Endospecy and Toxicolor tests and the results were compared. The mean±SD (pg/ml) of Endospecy and Toxicolor tests were 70.6±165.5 and 76.3±165.8, respectively. There was a significant correlation (Fig. 1., $r=0.949$, $p<0.0001$) between them (the correlation coefficient was obtained with Pearson's equation). However, endotoxin values of 40 specimens obtained by the Toxicolor test exceeded more than that of Endospecy. Especially, in sepsis case No. 18 (Table I), Toxicolor values in 6 of 7 specimens, exceeded 49.6±39.0 pg/ml more than that of Endospecy. Twenty-two specimens showed positive results (more than the cut-off value) in both tests, and 6 specimens showed positive results only by Toxicolor test (13.6, 11.8, 10.5, 53.6, 41.1, and 17.4 pg/ml), whereas one specimen showed positive results only by Endospecy (10.0 pg/ml).

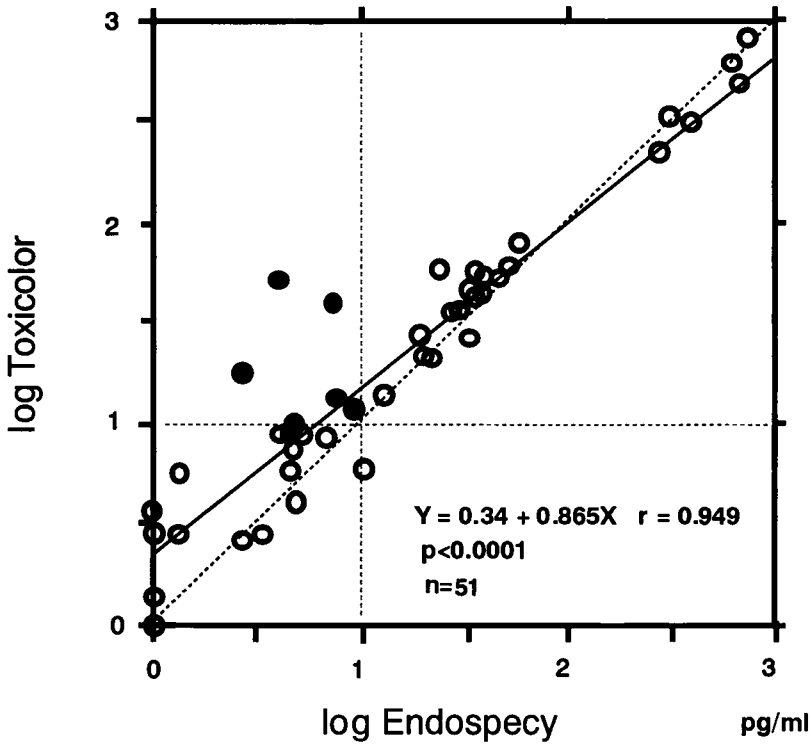


Fig. 1. Correlation between the endotoxin levels obtained by Toxicolor and Endospecy tests.

A diagonal line indicates Y=X. Closed circles indicate endotoxin values in which only Toxicolor values exceeded more than the cut-off value.

## DISCUSSION

Typhoid fever is characterized by bacteremia and the positive bacterial culture of *S. typhi* in blood, feces, the bile and urine at the feverish acute phase. In the present study, the isolation was attempted from blood and the bile, and *S. typhi*

was isolated from the bile but not from blood in 5 cases. The negative blood cultures in all cases can be explained by a late performance of the culture than by antibiotic therapy. *P. aeruginosa* was isolated from blood in 4 cases. This seemed to be due to the antibiotics used for these patients; *P. aeruginosa* is resistant to chloramphenicol or ampicillin.

Agglutination reaction (Widal test) is a useful tool for serological diagnosis. A rising titer of H or O antibodies in serial specimens of serum has been demonstrated in non-treated patients on antibiotics. Nowadays a single Widal test is performed in endemic areas at admission and before treatment with antibiotics. There are two reasons for a single Widal test: [14] 1) the patients in endemic areas show rapid and strong secondary response to the bacilli and 2) no further rise in titer is to be expected due to the early treatment with effective antibiotics. In the present study the cut-off titer was set at 1:160 as suggested by Rasaily R et al. [15]

On the contrary, endotoxemia was shown in 11 of 18 cases (61.1%). The incidence of endotoxemia seemed to be dependent upon the timing of collection of blood specimens for the assay; the early collection within 4 days shows relatively high incidence of endotoxemia (10/14, 71.4 %); in 4 cases, among 7 endotoxin-negative cases, specimens were collected at the convalescent phase. Endotoxemia means bacteremia or endotoxemia may be due to the release of endotoxin into the circulation from the outer membrane of destroyed bacteria by antibiotics. Thus, it was suggested that the early collection of blood specimens for endotoxin assay can produce better results as to diagnosis and the incidences of bacteremia and endotoxemia. In other words, endotoxin assay together with a bacterial culture seems to be a useful tool for the diagnosis of typhoid fever.

It is an acceptable idea that endotoxemia occurs in typhoid fever, but there was a report which showed noendotoxemia in typhoid fever. [2] However Adinolfi et al [1] reported that endotoxemia was found in all patients tested and discussed the relationship to liver dysfunction in patients with typhoid fever. In the present study, we detected plasma endotoxin with high incidence. The discrepancy is

thought to be due to the differences of limulus tests or pretreatment method for plasma or the timing of blood collection. The role of endotoxin in some symptoms has been considered, however, the studies on diseases in the human volunteer have minimized the role of endotoxin in its pathogenesis: [5] i. e., experimental typhoid fever in the endotoxin tolerant volunteer was clinically typical.

Two cases among typhoid fever cases expired from septic shock or the complication of DIC. It was thought that inflammatory cytokines induced by bacteria or bacterial-components might play a role in the pathological situations and possibly caused these deaths. [4] Thus, endotoxemia seemed to be one of the risk factors for a serious prognosis of infectious diseases.

It is unlikely that endotoxemia occurs in meningitis. However, it has been reported that the endotoxin content in cerebrospinal fluid is significantly high and is a good indicator of purulent meningitis due to gram-negative bacteria. [6]

The endotoxin-specific limulus test is the prerequisite for the endotoxin assay of clinical samples, because a conventional limulus test is responsible for not only endotoxin but also (1, 3)-β-D-glucan; endotoxin and glucan activated factor C pathway and factor G pathway, respectively. As glucan is a cell wall component of fungi, a conventional test shows positive results in the plasma of deep-seated mycosis. Glucan or glucan-like substances are found in patients on hemodialysis using a cellulosic membrane, or in cancer patients receiving anticancer polysaccharides. [13] The existence of unknown endogenous activity was reported to be the initiating of the factor G pathway in some clinical or normal conditions; e.g., in patient plasmas after a cardiopulmonary bypass operation [11] and after an esophageal varices operation, [8] in venous cord blood but not in maternal blood in normal delivery cases, [17] or in liver cirrhosis. [18] Therefore, use of a conventional limulus test may possibly lead to different conclusions in some clinical situations.

According to our results (K. Inada, et al, unpublished data), the endotoxin level by Endospecy test was 5.9±7.8 pg/ml, whereas it was 129.9±194.4 by Toxicolor test in 48 specimens from patients with infection due to *Staphylococci*, including

methicillin-resistant *Staphylococcus aureus.* Both values did not correlate with each other (r=0.139, p=0.3447). It was thought to be due to the fact that there were complicated pathophysiological conditions with underlying diseases, such as severe burns, multiple organ failure, leukemia, cancer, pneumonia and so on, and also in some patients with mycotic infection.

However, the difference between Toxicolor and Endospecy tests in the present results, is not remarkable and Toxicolor values exceeded in one case No. 18, in which the hospitalization days were extended up to one month and where the complicated pathophysiological situation seeme to be associated

These results suggest that measurement of plasma endotoxin in bacterial infection, including typhoid fever supports the diagnosis of bacteremia and might lead to precise information concerning the pathological condition.

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