

Serological approaches of Typhoid fever

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Abstrak

Berbagai pendekatan untuk diagnosis serologik demam tifoid telah mengungkapkan bahwa *S. typhi* mempunyai sedikit epitop spesifik yang berlokasi dalam berbagai komponen sel yang berbeda seperti protein flagel (flagellin), protein membran luar, O-polisakarida LPS, polisakarida Vi kapsul. Banyak upaya telah dilakukan dengan berbagai metoda untuk mendeteksi antigen spesifik dan antibodi anti *S. typhi* dalam serum dan antigen dalam urine pasien tifoid. Dibandingkan dengan tes konvensional Widal, upaya tersebut memperlihatkan kelebihan dalam spesivitas dan sensitivitas. Hasil kerja kami yang terbaru dengan flagellin *S. typhi* mengungkapkan bahwa epitop spesifik *S. typhi* yang berlokasi dalam 126 asam amino pada bagian tengah flagellin memberikan hasil yang menjanjikan ketika digunakan dalam ELISA untuk mendeteksi IgM spesifik dalam serum pasien tifoid akut. Penelitian ini meliputi kasus demam tifoid yang terbukti dengan kultur darah (92), salmonellosis non tifoid (31), bakteriemi gram negatif lain dan gram positif (34), 13 kasus demam berdarah dengue dan 116 kasus anak dan dewasa sehat. Lama tes adalah 8 jam dan tes tersebut memberikan spesivitas 98,97%, sensitivitas 83,70%, efektivitas 94,05% dengan nilai duga positif dan negatif masing-masing 97,45% dan 92,75%.

Abstract

Several approaches for serological diagnosis of typhoid fever have revealed that *S. typhi* has few specific epitopes which locate in different components of its cell, i.e. flagella protein (flagellin), outer membrane protein, O-polysaccharide of LPS, capsular Vi polysaccharide. Many attempts have been made by various means to detect both specific antigen and antibody of *S. typhi* in sera and antigen in urine of typhoid patients. In comparison with conventional Widal test, these attempts show more advantages in both specificity and sensitivity. Our recent work with flagellin of *S. typhi* reveals that the specific epitope of *S. typhi* which located within 126 amino acid in the mid portion of flagellin gives the promising result when used in ELISA detecting specific IgM in acute sera of typhoid patients. The study included hemoculture proven cases of typhoid fever (92), nontyphoid salmonellosis (31), other gram negative and positive bacteremia (34), 13 cases of dengue hemorrhagic fever and 116 cases of healthy children and adults. The assay time is 8 hours. It provides 98.97% specificity, 83.70% sensitivity, 94.05% efficacy with positive and negative predictive value of 97.45% and 92.75% respectively.

The best method for diagnosis of typhoid fever is isolation of *S. typhi* from bone marrow, blood, stool/rectal swab, and urine. Bone marrow culture has been proven to be the most sensitive diagnostic test but may not be feasible in all clinically suspected cases.¹ Blood, stool/rectal swab and urine culture have been found to detect *S. typhi* in moderate number of patients with typhoid fever². However, their usefulness is limited by many factors, for instances : stage of the disease, type of culture medium used, the length of the incubation period and previous antibiotic therapy. Thus, the chance to have false negative results are rather high.

Recently, a PCR based test was developed for the detection of *S. typhi* genome in the blood of typhoid patients³. It is a favorably specific test when the unique DNA sequences of *S. typhi* are used as primers.

However, this test has some disadvantages for the rural areas in developing countries, such as high cost per test, expensive instruments and required well trained personnel to handle the test. These disadvantages make PCR based test is not adopted for the routine laboratory service in developing countries yet.

The most commonly used diagnostic modality for typhoid fever is serological test which detected either *S. typhi* antigen or antibody to *S. typhi* in clinical specimens of typhoid patients. The test requires highly specific antibody or antigen to detect the corresponding antigen or antibody during proper stage of the disease. The conventional Widal test which is widely used for detect antibody employed whole bacterial cells agglutinate with patient's serum. It is a cheap assay, easy to perform, no need for expensive instrument and well trained personnel. A fourfold rising of antibody titer in paired sera is considered significant, but paired sera are seldom available. A single Widal test is not satisfactory in both specificity and sensitivity and is of dubious significance in the endemic area because of its cross-reactivity with nontyphoidal salmonellae and other enterobacteriaceae⁴.

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Thus, the disadvantage of Widal test in both specificity and sensitivity make many researchers seek for the new serological tests that provide better specificity and sensitivity.

Over the last decade, many attempts have been made to produce best specific antigen and antibody for use in serological assays with the hope that these assays would replace Widal test. The data from several reports have revealed that *S. typhi* has more than one specific epitope. These epitopes locate in different components of bacterial cell, i.e. flagellar protein (flagellin), outer membrane protein (OMP), O-polysaccharide of LPS, capsular Vi polysaccharide. The monoclonal antibodies (MAbs) against these epitopes have been established by many investigators. The unique epitopes of *S. typhi* have been identified and produced by either immunochemical or biotechnological means. The serological assays using those MAbs or unique epitopes of *S. typhi* have been set up. All assays have shown promising results such that they were better than Widal test in both specificity and sensitivity.

Flagella protein (Flagellin)

The flagellar filaments of Salmonella have 2 structurally and antigenically distinct phase-1 and phase-2 flagellins which express at different time in one bacterial cell. *S. typhi* express only phase-1 flagellin at all time⁵.

The phase-1 flagellin of *S. typhi* contains specific "d" antigen. This "d" antigen can be found in phase-1 flagellin of several salmonella serogroups, for example: serogroup B (i.e., *S. tanley*, *S. eppendorf*), serogroup C (i.e., *S. munche*n, *S. isangi*, *S. livingstone*), serogroup D (i.e., *S. typhi*, *S. strasbourg*), serogroup E (i.e., *S. birmingham*), etc. Among those salmonellae which have phase-1 d flagellin antigen, only *S. typhi* can cause typhoid fever in human.

The approaches to detect *S. typhi* phase-1 d flagellin antigen in sera of typhoid patients by monoclonal antibodies were reported in 1990 by Sadallah *et al*⁶ and Ekpo *et al*⁷. Both groups established monoclonal antibodies which were highly specific to *S. typhi*. These MAbs reacted with 52 KD salmonella phase-1 d flagellin⁶⁻⁸. With their MAbs, Sadallah and her colleagues developed a double antibody sandwich ELISA which was successfully used for laboratory diagnosis of typhoid fever. This assay could detect *S. typhi* flagellin in 95.5% of patients who had

hemoculture positive for *S. typhi*⁶. Double antibody sandwich ELISA using MAbs developed by Ekpo *et al* could detect 10 ng/ml of their specific antigen in the experimental model but failed to detect such antigen in patients' sera⁷.

Flagellin is protein which is a T-dependent antigen. The first antibody response to this type of antigen is IgM class which will switch to IgG class later during the course of disease and convalescent stage. In typhoid fever, detection of IgM specific to unique epitope of *S. typhi* flagellin in acute sera of patients should be beneficial for diagnosis of this disease.

The approach to identify unique epitope of *S. typhi* phase-1 d flagellin was attempted. Sukosol *et al* successfully obtained the nucleotide sequences of the whole *S. typhi* phase-1 d flagellin gene and noticed that the middle portion of this genome had lower degree of homology with the nucleotide sequences of phase-1 flagellin of other salmonellae⁹. Both ends of the gene were genetically deleted and the remaining middle portion containing nucleotides position 436-1339 was cloned into an expression vector pGEX-3X, then transformed into *E. coli* JM 101¹⁰. The protein product of recombinant gene was 302 amino acid in length, locating on the flagellin region IV-VI according to Wei and Joys classification¹¹. This protein was specific to MAbs established by Ekpo, *et al*⁷. Although this protein antigen could detect IgM in acute sera of typhoid patients, it also reacted with sera of few patients who were infected with salmonella non-phase-1 d flagellin as well as other gram negative bacteria. This finding indicated that the 302 amino acid antigen still contained common epitopes with other salmonellae and enterobacteriaceae.

Further approach to obtain a more specific antigen of *S. typhi* based on the phase-1 d flagellin was performed by Korbsrisate *et al*⁸. The hypervariable region IV, IV-VI and V-VI of *S. typhi* phase-1 d flagellin gene were amplified by PCR technique, and cloned into expression vector pGEX-2T, then transformed into *E. coli* TG1. MAbs specific to phase-1 d flagellin of *S. typhi* could react with protein products from hypervariable gene region IV and IV-VI but not with that from region V-VI. This finding indicated that the specific epitope of *S. typhi* was located in hypervariable region IV of phase-1 d flagellin.

The protein product from the hypervariable region IV is 126 amino acid in length and has the molecular weight of 13 KD (encoded by nucleotides position

532-909). Using this antigen, they have developed an indirect ELISA to detect IgM in acute sera of typhoid patients. The samples included 92 hemoculture proven cases of typhoid fever, 31 cases of non-typhoid salmonellosis, 34 cases of gram negative and gram positive bacteremia, 13 cases of dengue hemorrhagic fever, and 116 cases of healthy children and adults. The assay time is 8 hours. The result is shown in Table 1. It provides 98.97% specificity, 83.70% sensitivity, 94.05% efficacy, 97.45 and 92.75% positive and negative predictive values, respectively. They have concluded that although this unique epitope of *S. typhi* can be found in other salmonellae which have the same phase-1 d flagellin, this will be relatively little importance since human infected with those salmonellae except *S. typhi* is very rare and only *S. typhi* can cause clinical manifestation of typhoid fever. Thus, it will not be a problem for use this antigen in serodiagnosis of this disease.

Table 1 The findings of indirect ELISA for detection of IgM antibody to specific epitope on *S. typhi* phase-1 d flagellin.

Group	Total	ELISA	
		positive (%)	negative (%)
<i>S. typhi</i>	92	77 (83.7)	15 (16.3)
Non-typhoid Salmonella	31	0 (0)	31 (100)
Other bacteria: gram negative	19	0 (0)	19 (100)
gram positive	15	0 (0)	15 (100)
Dengue hemorrhagic fever	13	0 (0)	13 (100)
Normal subjects	116	2 (1.7)	114 (98.3)
Total	286	79	207

Outer membrane protein

The outer membrane is the outermost layer of cell envelope of gram-negative bacteria. It consists of lipopolysaccharide (LPS), lipoproteins and the outer membrane protein (OMP)¹².

In *S. typhi*, three major OMPs have been found and designated OmpC, OmpF and OmpA¹³. The antibody responses to OMP in typhoid patients have shown various degree of specificity in different OMP preparations, depended mainly on purity of the preparation. The detections of antibody to *S. typhi* OMPs have been used for serodiagnosis of typhoid fever.

Calderon *et al* were the first group to report the significance of specific IgG response to OMP in sera of Chilean typhoid patients¹⁴.

Ortiz *et al* used immunoblot for detection of class specific antibody response to OMPs in Mexican typhoid patients¹⁵.

Ismail *et al* reported the specific antibody response of typhoid patients to 50 KD OMP.¹⁶ Her group has developed a dot enzyme immunoassay using 50 KD OMP as antigen detected IgM and IgG antibodies in sera of Malaysian typhoid patients. The sensitivity and specificity of the assay were 90% and 91% respectively where either IgM or IgG was positive. The specificity of the assay increased to 100% when IgM was positive alone. For IgG positive alone, the specificity was much reduced¹⁷.

Verdugo-Rodriguez *et al* developed enzyme immunoassay using *S. typhi* OMP as antigen to detect anti-OMP antibodies in acute sera of typhoid patients. The assay gave a favorably result. They concluded that the EIA using OMP without LPS contamination might be useful for the diagnosis of typhoid fever¹⁸.

Nandakumar *et al* used porins of *S. typhi* detected anti-porins antibodies in typhoid patients by inhibition ELISA. The assay was promising for use as serodiagnosis of typhoid fever. The sensitivity, specificity, accuracy, positive and negative predictive value of this assay were 94.1%, 98.7%, 97.8%, 94.1% and 98.7% respectively¹⁹.

In reviewing those studies, there is still a question about the nature of specific epitopes in OMP of *S. typhi*. Further identification and purification of these epitopes would be beneficial for the study of immune responses as well as diagnostic modality in this disease.

O-polysaccharide of LPS

Lipopolysaccharide (LPS) is a unique constituent of the gram negative bacteria. It locates in the outermost layer of bacterial outer membrane. LPS is composed of three parts: the proximal hydrophobic lipid A region, the middle core polysaccharide region and the distal hydrophilic O antigen polysaccharide region¹². The core polysaccharide antigens are common in all salmonellae, the antigen specificity of each salmonella serogroup is located in the distal O antigen polysaccharide region. For salmonella serogroup D, the polysaccharide antigen 9 in the distal region of LPS has been reported to be specific for *S. typhi* as

well as other salmonella in this serogroup^{20,21}.

The monoclonal antibody specific for serogroup D salmonella antigen 9 was established by Chaicumpa, *et al.*²⁰ They used this MAb in an indirect ELISA and a dot blot immunoassay to detect the "antigen 9" in urine of typhoid patients. The assays provided 100% specificity and could detect as little as 7.8 ng of antigen in the urine. The sensitivity of the assay was increased from 65% when performed on a single specimen to 95% when serially collected urine samples from a patient were tested. They recommended both assays to be tested for antigen 9 in serially collected urine from typhoid patients, since *S. typhi* antigen is intermittently excreted in the urine of patients²¹.

The attempt to use anti-LPS of *S. typhi* for diagnosis of typhoid fever is reported by Shaheen, *et al.*²² They determined the serum levels of IgM, IgA, IgG and IgG subclass to *S. typhi* LPS in 40 typhoid patients and 66 patients for whom diseases other than typhoid were diagnosed. Using multivariate regression modeling, they suggested that detecting IgA, IgG and IgG2 to *S. typhi* LPS is of diagnostic value for typhoid fever.

Capsular Vi polysaccharide

The Vi polysaccharide is located on the outermost layer of bacterial outer membrane. This antigen is presented in limited strains of bacteria, i.e., *S. typhi*, *S. paratyphi C*, *C. freundii* and some strain of *S. dublins*²³. Among these 4 bacteria, only *S. typhi* and *S. paratyphi C* can cause clinical manifestations of typhoid fever. *S. paratyphi C* infection is very rare and responds to the same antibiotic treatment as *S. typhi*. Thus, Vi polysaccharide antigen is of interest to be used as another unique antigen of *S. typhi* for diagnosis of typhoid fever.

For detection of Vi polysaccharide antigen in blood and urine of typhoid patients, Rockhill, *et al.*²⁴ used slide coagglutination with commercial rabbit polyclonal anti-Vi antibody detected Vi antigen in urine of 96 clinically suspected cases of typhoid fever and 46 healthy control subjects. The assay detected Vi antigen in 97% of bacteriologically confirmed typhoid fever and 17% of healthy control. They claimed that this assay can be used for screening patients with suspected typhoid fever even the specificity and sensitivity are not very satisfied.

In contrary, Taylor, *et al.*²⁵ developed ELISA and slide coagglutination with rabbit polyclonal anti-*Citrobac-*

ter Vi antibody to detect Vi antigen in urine of patients. The results showed low specificity and sensitivity of the assays which suggested that the polyclonal Vi antibody reagents used in the assays might contain antibodies which cross-react with other bacteria. They concluded that the assays using polyclonal antibodies are of little value for the diagnosis of typhoid fever. The lack of specificity in these assays suggests that more specific antibodies directed to *S. typhi* Vi epitopes should be established.

The monoclonal antibodies to Vi polysaccharide antigen of *S. typhi* have been produced by Pongsunk, *et al.*²⁶ All these Mabs are highly specific to Vi antigen of *S. typhi* as well as Vi antigen of *S. paratyphi C*, *S. dublin* and *C. freundii*. The slide agglutination test using these Mabs for identification of *S. typhi* colony in primary bacterial culture has worked successfully but failed to detect Vi antigen in urine samples of typhoid patients. They concluded that the established Mabs can replace polyclonal anti-Vi antibodies that have been used routinely for identification of *S. typhi* in primary bacterial culture. For detection of Vi antigen in clinical specimens, more sensitive assay such as ELISA should be employed.

Vi polysaccharide is a T-independent antigen. The antibody responses to pure polysaccharide is IgM class only which will not stay last long after the disappearance of this antigen. Therefore, IgM anti-Vi antibody should disappear from the sera of previously *S. typhi* infected individuals and even in the late convalescent stage of the disease. The usefulness of detecting specific IgM to this type of antigen in acute sera of typhoid patients is recognized. We propose that the combined detection of IgM anti-phase-1 d flagellin and IgM anti-Vi antigen in acute sera of typhoid patients should be the most benefit for accurate diagnosis of this disease, since no salmonella which have phase-1 d flagellin has Vi polysaccharide, except *S. typhi*.

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