DOT ENZYME IMMUNOSORBENT ASSAY FOR THE SERODIAGNOSIS OF TYPHOID FEVER

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Abstract. A nitrocellulose membrane strip dotted with a specific 50 kDa outer membrane protein of *Salmonella typhi* was applied for the serodiagnosis of typhoid fever. Using horseradish peroxidase conjugated IgM and IgG antibodies with 4-chloronaphthol as substrate, antibodies in typhoid patients were clearly visualised as bluish purple dots while sera from patients with non-typhoid fevers gave negative results. The detection of specific IgM and IgG antibodies in typhoid patients suggest either recent or current infection. Combined with the high specificity, reliability and rapidity of the test, the dot EIA technique provides a simple and useful method for the serodiagnosis of typhoid using a single serum specimen.

INTRODUCTION

Enzyme immunoassays (EIA) using microtiter plates have been used in the diagnosis of typhoid fever (Barrett *et al,* 1982; Beasley and Weiss, 1981). Although the microtiter plate assays have the added advantage of processing many samples at a time with high sensitivity, they do have some limitations. Microtiter plate assays require expensive and special equipment which restricts its use to the larger hospitals and laboratories. Thus there is a need for a simple and rapid method to diagnose typhoid fever.

Dot EIA, which detects antibodies in patient sera to antigen dotted on nitrocellulose membrane has been applied in the diagnosis of microbial diseases (Itoh and Sato 1990; Oprandy *et al,* 1988). With this simple yet sensitive method, laboratory results can be interpreted without the use of special equipment.

The outer membrane proteins (OMPs) on the surfece of gram-negative bacteria have been considered as important antigens to induce host immune responses (Ortiz *et al,* 1989). Since cross-reactions among gram-negative OMPs can occur, purifications of antigenic components specific to *Salmonella typhi* must be made. We have previously determined that the 50 kDa OMP of *S. typhi* was specific for this bacterial species since it only reacted immunologically with typhoid sera (Ismail *et al,* 1991).

In this study, we attempted to test the specificity of the dot EIA as a diagnostic tool, to detect specific IgM and IgG antibodies in typhoid patients, using the 50 kDa OMP as the test antigen.

MATERIALS AND METHODS

Bacteria and antigen preparations

S. typhi USM1 was isolated from a patient with typhoid fever and has been maintained in our laboratory since 1987. Partially purified OMPs of *S. typhi* were obtained following the methodology as described by Schnaitman (1971). The concentrations of OMPs were determined using a colorimetric microassay procedure as recommended by Bio-Rad (Richmond, CA, USA) with bovine serum albumin as a standard.

The 50 kDa OMP of *S. typhi* was isolated via preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroelution. SDS-PAGE was performed under reducing conditions using the discontinuous buffer systems (Laemmli, 1970) with a vertical slab electrophoresis unit (Bio-Rad). The stacking and separating gels contained 4.5% and 9% acrylamide respectively.

Each preparative gel was loaded with 500 μg of OMP and was run at a constant current setting of 25 mA per plate at 4°C for 4 hours. The separated OMPs were stained with Coomassie blue, and the molecular weights were established with molecular weight markers of 14.4-94 kDa (Pharmacia, New Jersey, USA). The location of the 50 kDa OMP was determined and the band of interest was excised and minced to small pieces. The 50 kDa OMP was then eluted from the gel pieces via electroelution with a mini electroeluter unit (Bio-Rad) consisting of glass tubes fitted with membrane caps with a molecular weight cut-off of 15 kDa. Electroelution was performed at 10 mA per tube for 6 hours in an elution buffer containing Tris (0.3%), glycine (1.44%) and SDS (0.1%). The eluted protein was collected from the membrane caps and further concentrated by precipitation with two volumes of iced-cold ethanol, overnight, at 4°C. The precipitated proteins were pooled, resuspended in 0.03 M Tris and stored at -20°C until required. Protein concentration was determined using the colorimetric microassay procedure (Bio-Rad).

Sera samples

Sera specimens were obtained from 6 cases of culture confirmed typhoid patients, 2 cases of dengue, 2 cases of malaria, 2 cases of scrub typhus, 2 cases of serum hepatitis, 2 cases of paratyphoid A and 2 cases of paratyphoid B. Serum samples from uninfected healthy individuals were used as negative controls.

Dot EIA

A nitrocellulose membrane (Microfiltration system, CA, USA) (0.45 μm pore size), 0.5 cm by 1 cm, was used. 1 μl containing 0.03 μg of the extracted 50 kDa OMP was dotted onto the nitrocellulose using a microsyringe and allowed to dry. The strips were dipped into blocking buffer (3% skimmed milk, 0.9% NaCl, 10 mM Tris-HCl, pH 7.4), and placed on a rocker platform for 30 minutes, at room temperature. The blocked strips were rinsed 3 times for 15 minutes with 0.15 M NETG buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA and 0.25% gelatin), allowed to dry and kept at 4°C until use.

When performing EIA, the preblocked strips were labeled and placed in ice-cube trays. Each strip was then probed with one ml of 1 : 100 dilution of the different sera samples and incubated on a rocker platform for 1 hour at room temperature. Controls were performed with similar dilutions of normal serum and culture positive typhoid serum. The strips were then washed 3 times for 15 minutes with 1M NETG buffer (1M NaCl, 50 mM Tris-HCl, 5 mM EDTA and 0.25% gelatin) and further incubated on a rocker platform with 1 : 1,600 dilution of peroxidase conjugated anti-human IgG (Dakopatts, Glostrup, Denmark) or 1 : 800 dilution of anti-human IgM (Dakopatts) for 1 hour at room temperature. After washing the strips 3 times for 15 minutes in the blocking buffer, they were transferred into a color development solution (0.06% 4-chloro-naphthol, 0.015% H_2O_2, and 20% methanol) in blocking buffer. Color was allowed to develop for 15 minutes and the strips were rinsed in distilled water to stop the reaction. Sera containing either IgM or IgG antibodies specific to the 50 kDa typhoid antigen will give a bluish colored dot as intensive or more intensive than the positive control (culture positive typhoid serum).

RESULTS

As shown in Fig 1, the typhoid control serum gave a positive reaction for both IgM and IgG antibodies while the negative control (normal serum) gave only background staining. All 6 cases of culture-positive typhoid sera gave a strong positive reaction by dot EIA. Patients A and B gave only IgM positive reaction suggesting the acute stage of infection. Patients C and D were positive for both IgM and IgG suggesting the mid-stage of infection while patients E and F were positive only for IgG suggesting that they were already in the convalescent stage.

To evaluate the specificity of the test, sera from patients with non-typhoid fevers common in the region were also tested. No cross-reactions were observed since all the sera tested gave negative results.

DISCUSSION

The results obtained showed that the dot EIA using the 50 kDa OMP of *S. typhi* provides a specific laboratory test to diagnose typhoid fever

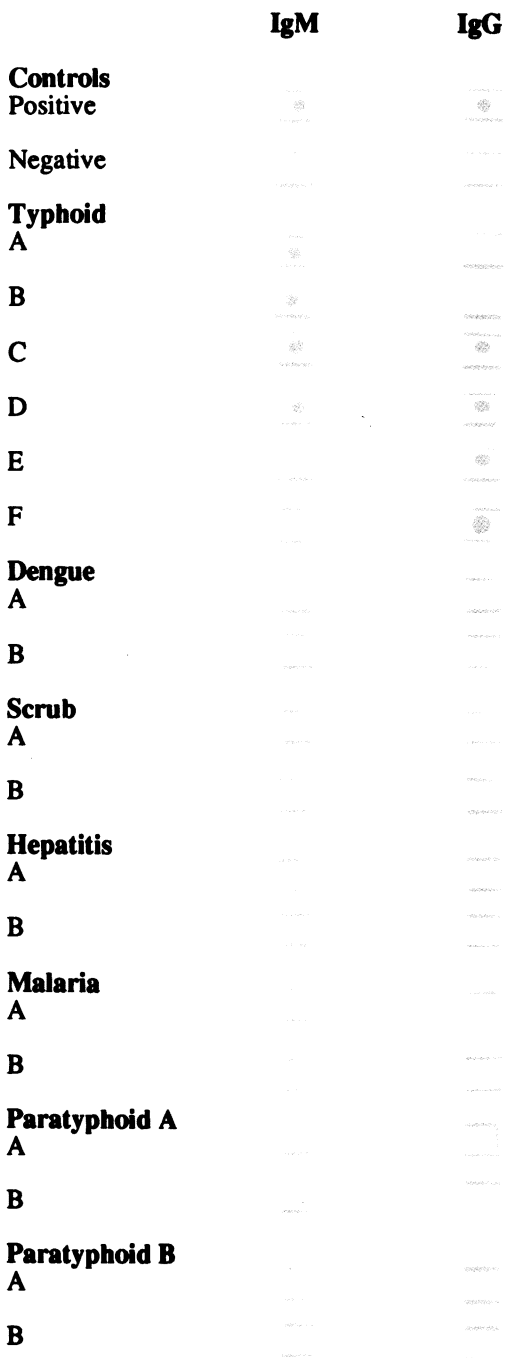

Fig 1—Dot EIA of typhoid and non-typhoid fevers. Dot EIA was carried out with 6 cases of culture positive typhoid sera (A to F), 2 cases of dengue (A and B), 2 cases of scrub typhus (A and B), 2 cases of hepatitis B (A and B), 2 cases of malaria (A and B), 2 cases of paratyphoid A (A and B) and 2 cases of paratyphoid B (A and B). Controls were performed with culture positive typhoid serum and normal serum. Sera containing either IgM or IgG antibodies specific to the 50 kDa typhoid antigen will give a bluish colored dot as intensive or more intensive than the positive control.

using a single serum specimen. The lack of cross-reaction with sera from non-typhoid fevers further enhanced the specificity of the test.

Another feature of the dot EIA is that it is cost-effective. Due to the small quantities of antigens and the size of the nitrocellulose used, the cost of the test per patient is essentially minimal. Furthermore, the lack of special equipment needed for this test, allows it to be used in the field and in the small district hospitals where culture facilities may not be available.

Antigens predotted and preblocked on nitrocellulose strips are stable for at least six months when kept at 4°C. When using such stored membranes, diagnosis of typhoid fever can be made within 3 hours upon receipt of patient serum specimen. This is a marked improvement over the conventional serological and cultural methods currently used in typhoid fever diagnosis. The availability of rapid diagnosis via dot EIA would allow the clinician to decide on prompt and effective antimicrobial therapy.

Addition of specific antigen dots for paratyphoid fevers on the membrane strips will increase the flexibility and usefullness of the dot EIA. Diagnosis of typhoid or paratyphoid fevers could easily be carried out at the same time. A comprehensive study to determine the specificity and reliability of dot EIA using the 50 kDa OMP of *S. typhi* compared to culture isolation and the Widal test is currently in progress in our laboratory.

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