

COMPARATIVE STUDY OF DIAGNOSTIC PROCEDURES IN *SALMONELLA* INFECTION, CAUSATIVE AGENT OF TYPHOID FEVER – AN OVERVIEW STUDY

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ABSTRACT

Typhoid fever is a prolonged and debilitating fever which is of major public health concern in many countries. Laboratory diagnosis of typhoid fever requires isolation and identification of *Salmonella enterica* serotype Typhi. In many areas where this disease is endemically, laboratory capability is limited. Recent advances in molecular immunology have led to the identification of sensitive and specific markers for typhoid fever and technology to manufacture practical and inexpensive kits for their rapid detection. Three commercial kits for serologic diagnosis of typhoid fever. Patients presenting with ≥ 4 days of fever were enrolled at two hospitals. Patients with serotype Typhi isolated from blood samples, and controls were patients with other laboratory-confirmed illnesses. This paper described following methods for diagnosis in case of typhoid fever, Laboratory analysis. (i) Blood culture (ii) Confirmation and antimicrobial susceptibility testing of isolates (iii) Investigation of stool culture (iv) Widal test. (v) Rapid tests (vi) A polymerase chain reaction (PCR)-based test.

Keywords: *Salmonella*, Typhi, Widal test, Blood culture

INTRODUCTION

Typhoid fever, caused by *Salmonella enterica* serotype Typhi, is a major cause of morbidity and mortality worldwide, causing an estimated 16.6 million new infections and 600,000 deaths each year. In India typhoid fever is highly endemic, with the southern provinces most heavily affected⁷. In a study conducted in Dong Thap Province in 1995 and 1996, the incidence of confirmed serotype Typhi infection was 198 per 100,000 for all ages. Isolation of serotype Typhi from blood, urine, or stool is the most reliable means of confirming an infection. However, this requires laboratory equipment and technical training that are beyond the means of most primary health care facilities in the developing world. Most serotype Typhi infections are diagnosed purely on clinical grounds and treated presumptively. As a result, the diagnosis may be delayed or missed while other febrile illnesses are considered, and patients without typhoid fever may receive unnecessary and inappropriate antimicrobial therapy¹⁰. Emerging drug resistance among circulating serotype Typhi strains in Vietnam and elsewhere has complicated the treatment of typhoid fever and heightened the need for rapid accurate diagnosis and the appropriate and selective use of antimicrobial agents to which the organism has thus far remained susceptible¹¹.

MATERIALS AND METHODS

Blood samples were centrifuged, and the serum was divided into aliquots and stored at -20°C . In order to minimize the degradation of the antibodies in the serum, the specimens were frozen immediately and remained frozen until the time of testing. At routine intervals, personnel from the Pasteur Institute retrieved the isolates and serum specimens from the hospitals; serum was stored at -70°C . All isolates were confirmed at the Pasteur Institute, and serum was reevaluated by using the Widal test. Serum specimens from all patients with a laboratory-confirmed illness were batched and shipped on ice every few months to the Centers for Disease Control and Prevention (CDC) in Atlanta, Ga., for further testing with the commercial assays. Patients with serotype Typhi isolated from blood were compared to patients with other laboratory-diagnosed pathogens by three commercial kits for rapid diagnosis of acute typhoid fever.

Laboratory analysis

(i) Blood culture. At Cai Lay Hospital, 5 ml of patient blood was added to blood culture medium (biphasic tryptic soy agar and brain

heart infusion broth with SPS [0.6 mg/ml]) supplied by the Pasteur Institute. The blood culture bottle was then incubated at 37°C for 24 h before being tilted so that the liquid flowed over the solid medium. The broth was subcultured on blood agar after 1, 2, 3, and 7 days, and the solid medium was subcultured any time there was a colony visible on the slant. Isolates were Gram stained and identified by standard biochemical methods. Serotyping was performed by using agglutination with *Salmonella* O, H, and Vi antisera. If there was no growth after 10 days, the culture was considered negative

(ii) Confirmation and antimicrobial susceptibility testing of isolates at the Pasteur Institute. The identification of suspect serotype Typhi isolates was confirmed at the Pasteur Institute by standard biochemical tests and *Salmonella* serotyping. Antimicrobial susceptibility testing was done by using the Kirby-Bauer disk diffusion method. The following antimicrobial agents (zone size for resistance) were used: ampicillin (≥ 17 mm), tetracycline (≥ 19 mm), chloramphenicol (≥ 18 mm), ceftriaxone (≥ 21 mm), ciprofloxacin (≥ 21 mm), ofloxacin (≥ 16 mm), norfloxacin (≥ 17 mm), nalidixic acid (≥ 19 mm), and gentamicin (≥ 15 mm).

Investigation of stool culture

A comparison of selenite F and the recommended selenite with mannitol (selenite M) was performed. Up to three stools were taken on admission or on consecutive days following admission, and approximately 1 gram of each stool was inoculated into 10 ml of either selenite F or selenite M (Oxoid, Basingstoke, UK). Stool specimens were inoculated into one or two bottles of each media. All media were prepared following the manufacturer's instructions. Each batch was quality controlled for pH and differential enrichment of *S. Typhi* in the presence of *Escherichia coli*. Broths were incubated for 24 hours at 37°C . The depth of the broth was 2.5 cm. A standard bacteriological loop was used to culture 5ml from the top of the broths onto MacConkey and XLD agars (Oxoid, Basingstoke, UK) after 18 -24 hours incubation. Isolates which had been previously grown from only one of the two selenite broths were tested for their ability to grow in both broths by inoculating 10^4 *S. Typhi* together with exactly 0.5 g of faeces from a healthy volunteer into 10 ml of each of the two broths. The stool from the healthy volunteer was tested for anti-*S. Typhi* activity by placing 1 g of faeces on a lawn of *S. Typhi* and 2 gm were inoculated into each of 20 ml of selenite F and selenite M to check for growth of *S. Typhi*. The selenite broths were subcultured at 18 and 24 hours.

Quantitative stool culture was carried out as follows: one gram of stool was emulsified in 10 ml of selenite M and left to stand for 30 seconds and 1mL and 10mL were then plated onto XLD agar using plastic disposable loops and spread for counting.

(iv) **Widal test**

Widal testing was done by using the Sanofi qualitative agglutination test kits (Bio-Rad) by two different methods. In both methods, serum was serially diluted, starting at 1/10, in physiological saline and then further diluted 1/10 in suspensions containing serotype Typhi O and H antigens, separately. Cai Lay Hospital used the rapid centrifugation technique in which the tubes were centrifuged at 3,000 rpm for 5 min. The precipitate was resuspended by tapping the bottom of the tube; if agglutination was visible, the results were considered positive. The Hospital for Tropical Diseases and the A polymerase chain reaction (PCR)-based test was developed for the detection of *Salmonella typhi* in the blood specimens from patients with typhoid fever. Blood culture, however, can detect only 45 to 70% of patients with typhoid fever, depending on the amount of blood sampled, the bacteremic level of *S. typhi*, the type of culture medium used, and the length of incubation period (6,7). In Korea, where typhoid fever is still common, diagnosis of many suspected cases on the basis of clinical findings cannot be confirmed because of negative cultures. The clinical usefulness of the culture method is further restricted because it takes at least 2 days until the identification of the organism. The development of a rapid and sensitive diagnostic method of typhoid fever, therefore, has a practical importance in endemic areas. Previously, a DNA probe specific to the Vi antigen of *S. typhi* had been used to detect the organism in the blood of patients with typhoid fever (9, 10, 12). This novel hybridization method, however, required concentration of bacteria* Corresponding author. from the blood samples and amplification of total bacterial DNA by overnight incubation of the bacteria on nylon filters to increase the sensitivity of the probe. This process of concentration was inevitable, because patients with typhoid fever usually have less than 15 *S. typhi* cells per ml of blood, and the probe cannot detect fewer than 500 bacteria. The problem of sensitivity of DNA probes could be circumvented by polymerase chain reaction (PCR), which can detect very small amounts of DNA by enzymatic amplification. PCR with the sequences of Vi antigen is not feasible, because the nucleotide sequence of this antigen has not been fully investigated. We report here the development of a PCR-base assay which can detect *S. typhi* DNA by amplification of the flagellin gene of *S. typhi* in the blood of typhoid patients.

PCR. PCR was carried out in three types of experiments. First, DNAs isolated from *Salmonella* spp. and other organisms were amplified to test the specificity of the PCR products. Second, the minimum detectable level by PCR was established by amplification of the serially diluted DNA from *S. typhi* ATCC 19430. To evaluate the influence of DNA from normally present leukocytes in the blood on the sensitivity of PCR, a known amount of DNA (2 p.g) from mononuclear cells was added to serially diluted DNA from *S. typhi*. Finally, PCR was performed with DNAs isolated from the blood of 12 actual typhoid patients, 10 patients with other febrile diseases, and 4 patients with suspected typhoid fever. The reaction mixture for the first round of PCR contained 2 p.g of extracted DNA, 25 pmol (each) of ST 1 and ST 2, 200 p,M (each) all four deoxyribonucleoside triphosphates, 0.625 U of Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany), and the standard PCR buffer (100 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, 0.1% gelatin [pH 8.3]) in a final volume of 25 µl. Amplification in an automated DNA thermal cycler (Hybaidd; Teddington, Middlesex, United Kingdom) consisted of 40 cycles at

Pasteur Institute used the classical technique with incubation in which the tubes were incubated in a 37°C water bath for 2 h for H suspensions and at room temperature overnight for O suspensions; if agglutination was visible, the results were considered positive.

(v) **Rapid tests.** Serum was evaluated by using the following three commercially available rapid diagnostic kits: Multi-Test Dip-S-Ticks (PANBIO INDX, Inc., Baltimore, Md.), TUBEX (IDL Biotech, Sollentuna, Sweden), and TyphiDot (Malaysian Biodiagnostic Research SDN BHD, Singapore, Malaysia). Briefly, the Multi-Test Dip-S-Ticks tests for five pathogens, including *Salmonella* serotype Typhi. The test is in a dipstick format that detects anti-O, anti-H, anti-Vi, IgM, or IgG antibodies in patient serum, plasma, or heparinized whole blood. Specimens were run according to the protocol listed on the packet inserts.

94°C for 1 min (denaturation), 57°C for 1 min and 15 s (annealing), and 72°C for 3 min (polymerization). After the reaction, 5 p.l of the amplified products of the first PCR was transferred to a second reaction mixture (20 p,l) containing 25 pmol (each) of ST 3 and ST 4 for the nested PCR. The nested PCR was performed for 40 cycles at 94°C for 1 min, 68°C for 1 min and 15 s, and 72°C for 3 min.

RESULT AND DISCUSSION

Currently, the laboratory diagnosis of typhoid fever is dependent upon either the isolation of *Salmonella enterica* subsp. *enterica* serotype Typhi from a clinical sample or the detection of raised titers of agglutinating serum antibodies against the lipopolysaccharide (LPS) (O) or flagellum (H) antigens of serotype Typhi (the Widal test). In this study, the serum antibody responses to the LPS and flagellum antigens of serotype Typhi were investigated with individuals from a region of Vietnam in which typhoid is endemic, and their usefulness for the diagnosis of typhoid fever was evaluated. Stool culture can be a useful aid to the diagnosis of acute typhoid and we have shown that the most commonly used selenite broth, selenite F, is as good as the recommended selenite M, thus removing the need for laboratories to carry both broths. A rarely investigated factor in stool culture is the volume of stool cultured. It is well reported that multiple samples of stool are needed for the isolation of *S. Typhi*. Our results confirm this: when three stools were cultured, 31.6% of patients were positive compared with only 13.4% from a single stool. In this study we also performed multiple cultures on single samples. By culturing 2 g of stool rather than the accepted 1g we increased the isolation rate by 10%.

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