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Detection of *Salmonella typhi* D, Vi, and d Antigens, by Slide Coagglutination, in Urine from Patients with Typhoid Fever

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Salmonella typhi antigens D, Vi, and d were detected in the urine of 59 out of 61 (97%) bacteriologically confirmed typhoid fever patients by slide coagglutination with monovalent antisera coupled to protein A-rich staphylococci. These antigens were also detected in the urine of an additional 22 patients, 16 of whom subsequently demonstrated seroconversion by *S. typhi* O antibody agglutination, but from whom the bacterium was not isolated. The remaining 13 patients had negative urine coagglutination results, no isolation of *S. typhi* from blood or stool specimens, and no demonstration of seroconversion. These results suggest that the method of slide coagglutination of urine can be used to screen patients with suspected typhoid fever with a high degree of reliability. The method may also have potential importance in the diagnosis of typhoid when the bacterium is not isolated.

Typhoid fever, although steadily diminishing in importance in the western world, remains a major cause of illness in developing countries. Epidemiological surveys of hospitalized febrile patients in Jakarta, Indonesia have shown that up to 25% of hospital admissions are due to typhoid fever (1). Admissions to the Special Hospital for Infectious Diseases in Jakarta consisted of 1,111 cases of confirmed and suspected typhoid during the years 1976 through 1978 and represented 51% of all hospital admissions for the observation of the fever (A. Moechtar Muhammad, The SEAMIC Conf. Semin. 1978).

Although typhoid fever does not present a well-defined clinical syndrome, its diagnosis in a hyperendemic focus may be made with a reasonable degree of certainty by an experienced clinician. In Indonesia most hospitalized patients with suspected typhoid fever are treated with oral chloramphenicol on an empirical basis after the collection of blood and stool specimens for bacteriological culture. From 1 to 2 days are required for even presumptive bacteriological results, and available serological methods of diagnosis require acute and convalescent sera and are often unreliable (16).

A number of other diseases may mimic typhoid fever (15) and require specific antimicrobial therapy. In addition, the agents causing the enteric-like illnesses may be resistant to chloramphenicol and other commonly available antimicrobial agents, particularly when the illnesses are caused by salmonellae other than *Salmonella typhi*. Therefore, in addition to exposing patients with suspected typhoid fever to an antimicrobial with significant potential toxicity (10, 18), such therapy may on occasion be inappropriate. Because of these factors it would be beneficial if a method were available that would allow the early and rapid demonstration of *S. typhi* as the causative agent before the institution of antimicrobial therapy.

This paper describes the detection of soluble *S. typhi* antigens in urine specimens collected from patients with typhoid fever. Somatic O groups D and Vi and flagella d antigens have all been detected with specific antisera coupled to protein A-rich staphylococci. The procedure can be performed within 30 min after receiving the specimen and provides a presumptive diagnosis with a high degree of certainty.

MATERIALS AND METHODS

Patients. The study group included a total of 142 individuals consisting of 96 hospitalized patients and 46 healthy control subjects. For inclusion in the study a patient had to have a history of fever of at least 1 week's duration, fever at admission, and at least two of the following: abdominal pain, mental confusion, constipation, hepatomegaly, or splenomegaly. Healthy subjects included 13 laboratory and 33 hospital employees. Patients were seen daily by one of us (L.W.R.), who obtained the blood cultures, rectal swabs, and urine specimens at the bedside.

Clinical specimens. Upon hospital admission, blood was drawn, four rectal swabs were obtained, and a urine specimen was collected. Thereafter, blood was drawn daily until the patient was afebrile. Rectal

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swabs were obtained at weekly intervals, and urine specimens were obtained daily until the patient was discharged.

The blood (3 ml) was added to 15 ml of 10% Oxgall (Oxoid Ltd., London, England) (11). Two rectal swabs were put into Amies transport medium (Difco Laboratories, Detroit, Mich.) and one into 3 ml each of mannitol selenite (9) and dulcitol selenite (14) salmonella enrichment broths (MSB and DSB) (Oxoid). The specimens were then transported to the laboratory within 1 h after collection, the blood culture and MSB and DSB were incubated for 18 to 24 h at 37°C, the urine was centrifuged at 3,000 × *g*, and the supernatant was collected and put into the refrigerator until tested. One swab from the Amies transport medium was put directly into MSB, and the second swab was used to inoculate MacConkey (MAC), deoxycholate-citrate-lactose-sucrose (DCLS), and salmonella-shigella (SS) agar media (Difco). This swab was then put into DSB. The sediment from the urine specimen was added to MSB. All cultures were incubated for 18 to 24 h at 37°C. After incubation, all MSB and DSB enrichment cultures were subcultured to MAC, DCLS, and SS agar plates. Blood cultures were subcultured daily, through 8 days or until positive for *S. typhi*, to MAC, DCLS, and SS agar media.

As many *S. typhi*-like colonies as were available to a maximum of 10 from each medium were subcultured to Kligler iron, lysine-iron, motility indole-ornithine, lysine decarboxylase, and urea agar media (Difco). Growth was taken from the Kligler iron agar slant, in the five-tube screen, that gave a presumptive *S. typhi* profile and was used to determine the serological reaction and confirm *S. typhi* (8).

Antisera. Monovalent D, Vi, and d antisera prepared in rabbits were obtained from a commercial source (Difco). Normal rabbit serum was prepared from animals housed at the Naval Medical Research Unit No. 2.

Preparation of the stabilized protein A-rich *Staphylococcus*. The Cowan 1 protein A-rich *Staphylococcus* strain (supplied by E. Edwards, Naval Research Laboratory, San Diego, Calif.) was grown confluently on Mueller-Hinton agar medium for 18 h at 37°C without added carbon dioxide. The cells were then harvested by emulsifying in 5 ml of phosphate-buffered saline (PBS: 0.03 M phosphate, 0.12 M NaCl [pH 7.2]) with a glass rod. Washing and heat stabilization procedures were done by the method of Edwards and Larson (7). Briefly, the harvest was washed three times with PBS, suspended in 0.5% formaldehyde in PBS for 3 h, again washed three times with PBS, and made up to a final 10% suspension in PBS. The suspension was then heated at 80°C for 1 h with constant stirring, washed three times in PBS, and stored as a 10% stabilized suspension in PBS at 5°C until coupled to the antiserum.

***Staphylococcus*-antibody coupling.** The method of Kronvall (12) was used to coat the cells. A 1-ml amount of the 10% *Staphylococcus* preparation was mixed with 0.1 ml each of monovalent D, Vi, and d *Salmonella* antisera and normal rabbit serum. The individual mixtures were left at room temperature for 3 h and gently agitated at half-hour intervals. Each lot was used without further washing. Before use, 1 ml of each sensitized suspension was diluted with 9 ml of PBS. These final suspensions were used as the reagents, hereafter called D-COAG, Vi-COAG, d-COAG, and NRS-COAG.

Urine COAG test. For the urine coagglutination (COAG) test, one drop (50 μl) of the supernatant was added to each of five ringed areas on a glass slide. D-, Vi-, and d-COAG (50 μl) were added to the first three samples, and NRS-COAG and stabilized *Staphylococcus* were added only to the last two samples to serve as negative controls. The urine and reagent were mixed with an applicator stick, and the slide was rotated continuously by hand for 5 min. The time required for distinct agglutination was noted.

Widal serology. The Widal method of slide agglutination was used to determine serum antibody levels of *Salmonella* group D somatic O antigens (3). Acute and convalescent sera were obtained at least 2 weeks apart. Seroconversion was considered to have taken place if a greater than fourfold increase in the antibody was demonstrated between the acute and convalescent specimens.

RESULTS

Sixty-one patients (64%) meeting the clinical criteria required for inclusion in the study had *S. typhi* isolated from either the blood (35 out of 61, 57%) or stool (45 out of 61, 74%) specimens collected upon hospital admission. No *S. typhi* was isolated from urine sediments. Admission urine specimens from 59 out of 61 (97%) of these patients gave positive COAG reactions. All reactions were strong (2 to 4+) and occurred within 30 s to 1 min. The Vi-COAG reaction was predominantly stronger by 1+ than the D- or d-COAG reaction and occurred first. There were no instances in which all three reagents did not give a COAG reaction with the soluble *S. typhi* antigens present in the positive urine specimens. In addition, NRS-COAG never reacted with the urine components to give agglutination. The urine COAG test was negative for 2 out of 61 (3%) of the patients from whom *S. typhi* was isolated from the rectal swab cultures.

Urine specimens obtained from 22 out of 35 (63%) of the patients gave a positive COAG reaction, although the bacterium was never successfully isolated from the blood, rectal swab, or urine. Sixteen of these patients later demonstrated seroconversion to *Salmonella* group D somatic O antigens, four did not seroconvert, and convalescent sera were not available for testing from two patients. The remaining urine specimens, from 13 out of 35 (37%) of the patients from whom *S. typhi* was not isolated and who failed to seroconvert, were COAG negative.

Soluble D, Vi, and d antigens were detected in the urine of 8 of the 46 control subjects. None had a previous illness suggestive of typhoid. The eight positive individuals were paramedical hospital personnel who regularly attended typhoid

fever patients or were in close contact with body excretions from such patients. We attempted to ascertain if these eight individuals harbored *S. typhi*. Sera obtained for Widal agglutination were uniformly negative. Routine rectal swab cultures were made on three separate occasions, and in two instances nontyphoid salmonellae were isolated (*Salmonella paratyphi-B* and *Salmonella weltevreden*) from different subjects. One of the control subjects from this group, however, developed bacteriologically confirmed typhoid fever 2 weeks after the initial positive urine COAG test.

DISCUSSION

Because typhoid fever does not present a distinct clinical syndrome and has a high morbidity associated with delay in therapy and because major complications frequently occur in the untreated disease, a simple, rapid test which would allow the early presumptive diagnosis would be beneficial. We have previously demonstrated the usefulness of the COAG of MSB and DSB inoculated with feces from patients with known typhoid fever (unpublished data), and the success of this method prompted us to look for *S. typhi* D, Vi, and d antigens in urine specimens.

Positive D-, V-, and d-COAG occurred in 59 (97%) urine specimens from 61 patients with culture-proven typhoid fever. Soluble D, Vi, and d antigens were detected in the initial urine specimens obtained at hospital admission in almost every instance. The urine was also COAG positive from 22 patients from whom *S. typhi* was not isolated but who demonstrated fourfold or greater rises in the *Salmonella* group D somatic O antibody. Although all of these 22 patients were felt to have typical clinical signs and symptoms suggestive of typhoid, the lack of bacteriological confirmation undoubtedly reflected prehospital antibiotic therapy which occurred in >90% of these patients.

The urine COAG test was negative for two patients from whom *S. typhi* was isolated from the rectal swab cultures. Reduced renal clearance may have been a factor as both patients had elevated serum creatinine levels. A reduction in the glomerular filtration rate and reduced levels of antigen excretion may have resulted in antigen levels below the detection limits of the test.

Early in the course of the study, false-positive reactions randomly occurred with unsensitized staphylococci and NRS-COAG as the negative controls. Microscopic examination of the urine sediments suggested that leukocytes could be coagglutinating with these reagents as the positive control urine specimens appeared to contain more leukocytes than the negative control specimens. Centrifugation eliminated this artifact. We also found that a preadsorption step of adding native or stabilized protein A-rich staphylococci or NRS-COAG reagent to the urine to eliminate nonspecific agglutination greatly reduced the magnitude of the agglutination when the monovalent antisera were later added.

Eight control subjects had positive urine COAG tests. Two of these subjects were found, after repeated stool cultures, to harbor salmonellae (including *S. typhi* from one who later developed typhoid fever). Therefore, these actually may not represent bona fide false-positive reactions. Although the possibility of a serological cross-reaction between *S. typhi* and other *Salmonella* and members of the *Enterobacteriaceae* exists, further studies are needed to determine what effect this may have when the urine COAG test is used. Each monovalent antiserum reagent gave distinct agglutination in every instance. It is certainly conceivable that the control subjects exhibiting positive reactions were chronic carriers of *S. typhi*. Their occupations, involving close daily contact with typhoid fever patients and with the excretions and fomites from such patients, placed them at high risk for the acquisition of *S. typhi*. The bacteriological recovery of this organism from long-term carriers is notoriously difficult as stool excretion may only be intermittent (4, 15).

The technique of bacterial COAG has been successfully applied in the laboratory identification of various bacterial species (5–7, 12). Recent reports have also dealt with the identification of organisms directly from clinical material by this method (13, 17). The results of this study strongly suggest the additional usefulness of the COAG test as an adjunct in the diagnosis of typhoid fever. Cultures require lengthy laboratory processing, with negative results in >30% of instances. In addition, serological studies, such as the Widal, are dependent upon the previous exposure and treatment of the patient. However, the COAG test is rapid and may offer a sensitive and specific method for the early presumptive diagnosis of typhoid fever.

Our study group consisted of a select group of persons from a high-risk population, and, therefore, the test sensitivity (97%) may be a reflection of this condition (2). In addition, the specificity could suggest that the COAG test would be inappropriate when testing persons in a high-risk population as, according to the results, 63 out of every 100 nontyphoid fever patients would be presumptively diagnosed as having the disease. If the 16 patients that seroconverted and

had positive urine COAG tests are included with the bacteriologically documented typhoid patients, then the figure is reduced to 17 out of every 100 giving false-positive reactions. It may follow that the test would be even more specific in a nonendemic area because of less likelihood for exposures and subclinical infections that might result in a positive test.

The specificity of 83% in the control group further supports the idea that exposure and subclinical infections or both may be reflected in the positive COAG tests as all of the positive results came from persons who regularly attended typhoid fever patients. Here again the results suggest that 17 out of 100 healthy persons would be diagnosed as having typhoid fever. If the control group had been reserved for those persons only at the Naval Medical Research Unit, representing a relatively low-risk population, then there would not have been any false-positive reactions, and the specificity would have been 100%. Until it is determined whether the apparent low specificity is real or is an artifact, based on bacteriological confirmation, the COAG test appears to merit further study as a diagnostic aid, especially as *S. typhi* was not isolated in this study from 22 patients who had a clinical diagnosis of typhoid fever, a positive COAG test, and who, in 16 cases, later developed the antibody to *Salmonella* O antigens. Also, one out of eight in the control group with a positive COAG test later developed typhoid fever, and the incubation period was such that the COAG test may have detected the antigen during the prodromal stage.

Studies are currently under way to determine the following: (i) the effects that other non-*S. typhi* infections have on the sensitivity and specificity of the COAG reaction; (ii) how long the antigens can be detected after the institution of effective antimicrobial therapy; (iii) how levels of the antigens (or of the intensity and duration of COAG) vary in relation to the severity of the disease, including the carrier state; (iv) whether the antigens can be detected during the prodromal stage; and (v) whether the COAG test can be used reliably to presumptively diagnose typhoid fever in the absence of other laboratory confirmation.

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