RAPID DIAGNOSIS OF TYPHOID FEVER BY ENZYME-LINKED IMMUNOSORBENT ASSAY DETECTION OF SALMONELLA SEROTYPE TYPHI ANTIGENS IN URINE

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Abstract. We developed and evaluated a enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies to capture somatic antigen 9 (O9), flagellar antigen d (Hd), and the Vi capsular polysaccharide antigen (Vi) from the urine of persons with and without typhoid fever. Sequential urine samples were collected from 44 patients with blood culture-confirmed typhoid fever and from two control groups. The first control group included patients with brucellosis (n = 12) and those with clinically diagnosed, non-typhoid, acute, febrile illness (n = 27). The second control group was a sample of healthy volunteer laboratory workers (n = 11). When assessed relative to date of fever onset, sensitivity was highest during the first week for all three antigens: Vi was detected in the urine of nine (100%) patients, O9 in 4 (44%) patients, and Hd in 4 (44%) patients. Sequential testing of two urine samples from the same patient improved test sensitivity. Combined testing for Vi with O9 and Hd produced a trend towards increased sensitivity without compromising specificity. The specificity for Vi exceeded 90% when assessed among both febrile and healthy control subjects, but was only 25% when assessed among patients with brucellosis. Detection of urinary Vi antigen with this ELISA shows promise for the diagnosis of typhoid fever, particularly when used within the first week after fever onset. However, positive reactions for Vi antigen in patients with brucellosis must be understood before urinary Vi antigen detection can be developed further as a useful rapid diagnostic test.

INTRODUCTION

Typhoid fever is an acute, generalized infection of the reticuloendothelial system. It is caused by Salmonella enterica subspecies enterica serotype Typhi (Salmonella Typhi), which causes an estimated 16 million illnesses and 600,000 deaths worldwide each year. Typhoid fever is endemic in Egypt. Population-based studies indicate that typhoid fever incidence is 10–100/100,000/year, with an annual peak in August (national passive typhoid fever surveillance system, Egypt Ministry of Health and Population, unpublished data). Typhoid fever is difficult to distinguish clinically from other causes of prolonged fever. Countries with high rates of disease often lack the capacity to perform blood cultures to confirm the diagnosis, which may delay the initiation of antimicrobial therapy and lead to increased mortality in patients with typhoid fever. Therefore, developing an inexpensive and rapid diagnostic test for typhoid fever that is both sensitive and specific has become a public health priority. Serologic assays have potential for manufacture on platforms suited to use in developing countries.

The history of serologic approaches to the diagnosis of typhoid fever spans more than a century, beginning with the development of the Widal agglutination test. Because it is simple and inexpensive, the Widal test has gained widespread use despite shortcomings of both sensitivity and specificity that compromise its utility as a diagnostic test. Enzyme-linked immunosorbent assay (ELISA) methods have been used to detect antibody to various infectious agents, including Salmonella Typhi. However, for typhoid fever, these methods have been hampered by similar limitations of sensitivity and specificity that occur with the use of the Widal agglutination test.

Consequently, efforts have been made to detect Salmonella Typhi antigens from clinical samples from patients with typhoid fever by antigen-capture ELISA. Polyclonal capture antibodies have been used to detect bacterial antigens of S. Typhi from various body fluids, of which urine appears to provide the greatest sensitivity. However, antigen-capture ELISA has been limited by poor specificity. The presence of a non-specific, cross-reacting substance from the urine of febrile patients or the limitations of polyclonal antibodies have been offered as explanations. Recently, monoclonal antibodies have been developed that might mitigate the cross-reactivity inherent with the use of polyclonal antibodies for diagnosis of typhoid fever.

We developed and evaluated an ELISA using monoclonal antibodies in an attempt to capture somatic antigen 9 (O9), flagellar antigen d (Hd), and the Vi capsular polysaccharide antigen (Vi) from the urine of persons with and without blood culture-confirmed typhoid fever.

MATERIALS AND METHODS

This study was reviewed and approved by the Institutional Review Boards of the Naval Medical Research Unit No. 3, the United States Centers for Disease Control and Prevention (CDC), and the Egyptian Ministry of Health and Population. In addition, informed consent was obtained from all human adult participants and from parents or legal guardians of minors.

Case selection. Urine samples were collected from patients admitted to Abbasia Fever Hospital (Cairo, Egypt) from June 2000 through September 2001 who had positive blood cultures for Salmonella Typhi.

Control selection. Urine samples were collected from two control groups. The first control group included 39 febrile patients. Blood cultures from these patients were negative for S. Typhi. Brucella spp. was isolated from the blood of 12 (31%) of these patients. The remaining 27 (69%) patients had clinically diagnosed non-typhoid acute febrile illness. The second control group was a sample of healthy volunteer laboratory workers who donated urine for the study.
Specimen handling. Urine samples were collected on hospital admission from each patient. Subsequently, urine was collected daily until the time of discharge. Urine samples were stored within three hours of collection at \(-20^\circ C\). All urine samples from typhoid fever patients were classified in time relative to the date of fever onset and relative to the date of collection of the positive blood culture. The first urine sample collected following hospital admission from patients with blood culture-confirmed typhoid fever was classified as the baseline urine sample.

Blood cultures. Blood cultures were conducted using the Phase2™ bi-phasic blood culture system (PML Microbiologicales, Wilsonville, OR). Bottles were incubated for 14 days at 35°C and observed daily for signs of microbial growth. Growth in broth or on agar paddles was examined by Gram’s stain and was subcultured onto solid media. Colonies were identified by standard methods.28

Enzyme-linked immunosorbent assays. Sandwich ELISAs were developed for the detection of *Salmonella* serotype Typhi O9, Hd, and Vi antigens. Purified bacterial antigens including lipopolysaccharide of somatic (O9) antigen (Sigma-Aldrich Chemical Company, St. Louis, MO), *Salmonella* serotype Eschberg flagellar (Hd) antigen (CDC, Atlanta, GA), and Vi capsular antigen (CDC) were used to develop and characterize the different ELISAs for antigen detection. Each step of the assay, including incubation times and dilutions, were optimized by checkerboard titration. The different assays are described in detail as follows.

Polystyrene microtiter plates (Immunlon 2; Dynex Plastics, Chantilly, VA) were coated with 100 μL of monoclonal antibody (10 μg/mL in phosphate-buffered saline [PBS]) to somatic (O9), flagellar (Hd) (courtesy of Paul Duffey, California State Health Department Laboratory, Berkley, CA), and capsular (Vi) antigens (Sinfin, Inc., Berlin, Germany). The plates were incubated for either three hours at 37°C or overnight at 4°C and were then washed with PBS, 0.1% Tween 20. The plates were blocked using PBS containing 2% bovine serum albumin (BSA) and 0.2% Tween 20 (200 μL/well) and incubated at 37°C for one hour. Urine samples were loaded onto the plates (100 μL/well) and each sample was tested in duplicate. The purified bacterial antigens described earlier and PBS were used as assay-positive and -negative controls, respectively, when testing urine samples. Plates were incubated for 90 minutes at 37°C. Rabbit polyclonal antibody (100 μL/well) (CDC) for each antigen was used as a second antibody in the assay. Dilutions were 1:500 for polyclonal somatic antibody, 1:2,000 for polyclonal flagellar antibody, and 1:320 for the polyclonal Vi antibody. Phosphate-buffered saline, 0.2% BSA was used as the diluent. Plates were incubated for one hour at 37°C and were then washed with PBS, 0.1% Tween 20. Goat anti-rabbit IgG conjugated with peroxidase (100 μL/well; Bio-Rad Laboratories, Hercules, CA) was used as the detecting reagent. Dilution ratios of 1:10,000 and 1:5,000 were used for the somatic assay and the flagellar and Vi assays, respectively, using PBS, 0.2% Tween 20, 0.2% BSA as the diluent. Plates were incubated for 30 minutes at 37°C and were then washed with PBS, 0.1% Tween 20. Orthophenylenediamine dihydrochloride (Sigma FAST™; Sigma-Aldrich Chemical Company) was used as the substrate. One hundred microliters was added to each well and incubated at room temperature for 30 minutes in the dark. The reaction was stopped using of 4 N H₂SO₄ (25 μL/well) and the developed absorbance was read using an automatic ELISA reader at 490 nm and 550 nm as main and reference filters, respectively.

Statistical methods. The ELISA optical density (OD) values that maximally separated typhoid patients from control subjects (cut-off) were established based on the mean reading of the healthy volunteer control samples plus two standard deviations. Sensitivity and specificity were determined using baseline urine samples. To plot time curves for each urinary antigen, urinary O9, Hd, and Vi results were aggregated by day relative to the day of collection of the positive blood culture.

Sensitivity was initially evaluated using baseline urine samples from patients with blood culture-confirmed typhoid fever. For these samples, sensitivity was assessed first for O9, Hd, and Vi antigens individually and then for Vi antigen in combination with O9, Hd, or both. Changes in sensitivity for antigen combinations with Vi were assessed by comparison with testing Vi antigen alone using the chi-square test for proportion. Sensitivity was further assessed for urine samples from patients with blood culture-confirmed typhoid fever by week from date of fever onset for O9, Hd, and Vi antigens individually. Sensitivity for detection of Vi, O9, and Hd antigens from multiple urine samples (i.e., four or more samples) was assessed among patients with blood culture-confirmed typhoid fever.

Specificity was assessed using baseline urine samples from patients with non-typhoid febrile illness, including blood culture-confirmed brucellosis, other acute febrile illness, and healthy controls. Specificity was assessed initially for Vi, O9, and Hd antigens alone and then for Vi antigen in combination with O9, Hd, or both. Changes in specificity for antigen combinations with Vi were assessed by comparison with testing Vi antigen alone using the chi-square test for proportion.

The impact of antimicrobial therapy on the persistence of *Salmonella* Typhi antigens in urine was assessed among patients with blood culture-confirmed typhoid fever by studying antigen detection in sequential urine samples after the date of collection of the first positive blood culture.

RESULTS

Characteristics of study subjects. Urine samples were obtained from consecutive patients with blood culture-confirmed typhoid fever (n = 44) identified among febrile patients admitted to Abbassia Fever Hospital in Cairo, Egypt from June 2000 through September 2001. Urine from febrile control subjects was obtained among consecutive patients with either blood culture-confirmed brucellosis (n = 12) or a febrile illness other than typhoid fever (n = 27) identified from the same institution. The first urine sample was collected before the administration of the first in-patient dose of antimicrobials. In addition, urine from afebrile control subjects was obtained among healthy volunteer laboratory workers (n = 11).

Detection of *S. Typhi* antigens in urine. Detection of *S. Typhi* antigens in urine was evaluated in relation to several parameters: baseline urine sample, date of fever onset, and after administration of antimicrobial agents.

Baseline sample. When analysis was restricted to the baseline urine sample (i.e., the first urine sample collected after hospital admission from patients with blood culture-con-
firmed typhoid fever), Vi antigen was detected in 32 (73%), O9 antigen in 19 (43%), and Hd antigen in 16 (37%) of 44 patients. Mean, standard deviation, and ranges of OD values for the study groups are shown in Table 1. When positive patients were further categorized as weakly, moderately, and strongly positive, it was observed that of the 32 Vi-positive baseline urine samples, 6 (19%) were classified as weakly positive (OD range $0.20 - 0.30$), 3 (9%) as moderately positive (OD $0.31 - 0.60$), and 23 (72%) as strongly positive (OD $> 0.61$). Of the 19 O9-positive baseline urine samples, 10 (53%) were classified as weakly positive, 7 (37%) as moderately positive, and none as strongly positive. Of the 16 Hd-positive baseline urine samples, 8 (50%) were classified as weakly positive, 7 (44%) as moderately positive, and 1 (6%) as strongly positive.

**Date of fever onset.** To assess the optimal timing for collection of urine samples to identify patients with typhoid fever using our assays, we evaluated the detection of antigens in samples collected during weeks 1 through 4 after the fever onset date. The urine samples tested included the baseline and follow-up samples from each case. The number of follow-up samples collected from each patient varied from 4 to 10. This means that all urine samples collected from each patient were related to the fever onset date for that patient. During week 1, Vi was detected in the urine of all nine patients (100%); both O9 and Hd were detected in four (44%) of nine patients. During week 2, Vi was detected in the urine of 71 (70%) of 102 patients, O9 in 34 (33%) of 102 patients, and Hd in 26 (26%) of 101 patients. During week 3, Vi was detected in the urine of 58 (62%) of 93 patients, O9 in 31 (36%) of 87 patients, and Hd in 25 (29%) of 86 patients. During week 4, Vi was detected in the urine of 16 (67%), O9 in six (25%), and Hd in four (20%) of 24 patients (Figure 1).

**Impact of administration of antimicrobial agents.** To evaluate the persistence of Vi, O9, and Hd antigens after initiation of specific antimicrobial therapy, we studied urine samples day by day after collection of the diagnostic blood culture. Urinary Vi antigen was detected in 17 (81%) of 21 patients whose samples were tested on the day of collection of the positive blood culture, and in 18 (86%) of 21 patients whose urine was tested on the day following collection of the positive blood culture. Although detection of urinary Vi antigen decreased thereafter, Vi antigen was still detectable from one (25%) of four patients tested 10 days after collection of the positive blood culture (Figure 2). Urinary O9 antigen was detected in seven (33%) of 21 patients whose specimen was tested on the day of collection of the positive blood culture, and in 18 (86%) of 21 patients whose urine was tested on the day following collection of the positive blood culture. Urinary O9 antigen detection peaked on day 4, with 16 (59%) of 27 patients testing positive. Urinary Hd antigen was detected in seven (33%) of 21 patients tested on the day of collection of the positive blood culture, and in seven (33%) of 21 patients tested on the day following collection of the positive blood culture.

### Table 1

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Parameter</th>
<th>Typhoid (n = 44)</th>
<th>Non-typhoid (n = 39)</th>
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<tr>
<td>Vi</td>
<td>OD ± SD</td>
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<td></td>
<td>No. (%)</td>
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<td>9 (75)</td>
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<td>O9</td>
<td>OD ± SD</td>
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<td>0.12 ± 0.13</td>
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<tr>
<td></td>
<td>Range</td>
<td>0.0–0.9</td>
<td>0.0–0.1</td>
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<tr>
<td></td>
<td>No. (%)</td>
<td>19 (44)</td>
<td>2 (17)</td>
</tr>
<tr>
<td>Hd</td>
<td>OD ± SD</td>
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<td>0.06 ± 0.07</td>
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<tr>
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<td>Range</td>
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</tr>
<tr>
<td></td>
<td>No. (%)</td>
<td>16 (37)</td>
<td>1 (8)</td>
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</table>

* OD ± SD = Mean optical density value ± standard deviation.

**FIGURE 1.** Detection of urinary Vi capsular, O9 somatic, and Hd flagellar antigen among patients with culture-confirmed typhoid fever in relation to date of fever onset.

**FIGURE 2.** Persistence of urinary Vi capsular, O9 somatic, and Hd flagellar antigen among patients with culture-confirmed typhoid fever in relation to date of collection of positive blood culture.
Hd antigen detection peaked on day 4, with 13 (48%) of 27 patients testing positive. Urinary O9 and Hd antigen were undetectable in the urine of all patients by day 10 (Figure 2).

Assay sensitivity. In addition to evaluating sensitivity of testing baseline and subsequent urine samples, we studied the impact of testing combinations of antigens and of testing multiple urine samples from the same patient on sensitivity. The results of testing various antigens in combination are summarized in Table 2. Of 44 samples, Vi or O9 was detected in 35 (80%) ($P > 0.05$ when compared with Vi alone), Vi or Hd in 34 (77%) ($P > 0.05$), and Vi or O9 or Hd in 36 (82%) ($P > 0.05$). The results of testing multiple sequential daily urine samples from the same patient are summarized in Table 3. Vi was detected in the urine of 22 (73%) of 30 patients after one sample was collected, in 28 (93%) of 30 after two samples were collected, in 29 (97%) of 30 after three samples were collected, and in 29 (97%) of 30 patients after four samples were collected. O9 was detected in the urine of 12 (40%) of 30 patients after one sample and in 16 (53%) of 30 patients who had two, three, or four samples collected. Hd was detected in the urine of 10 (33%) of 30 patients after the collection of one sample and in 16 (53%) of 30 patients who had two, three, or four samples collected.

Assay specificity. For the baseline urine samples of patients with blood culture-confirmed brucellosis, Vi was detected in the urine of nine (75%) of 12 patients, O9 antigen in the urine of two (17%) of 12 patients, and Hd antigen in the urine of one (8%) of 12 patients. For the baseline samples of patients with febrile illnesses, Vi was detected in the urine of two (7%) of 27 patients, O9 antigen in the urine of zero (0%) of 27 patients, and Hd antigen in the urine of zero (0%) of 27 patients. For the baseline samples of healthy control-patients, Vi, O9, and Hd antigen were detected in zero (0%) of 11 tested (Table 1). Specificity was also evaluated for Vi antigen in combination with O9 antigen, Hd antigen, or both. Combining testing with O9, Hd, or both did not reduce the specificity of the assay compared with testing Vi antigen alone (Table 2).

### DISCUSSION

In an effort to develop a rapid, reliable, specific, and sensitive test for the diagnosis of typhoid fever, we designed and evaluated sandwich ELISAs using monoclonal antibodies to capture S. Typhi antigens in urine samples of typhoid patients. Urine was selected because it is easy to obtain and would be suitable for mass screening studies.

When the baseline urine samples collected from typhoid patients were tested, we found a low sensitivity in culture confirmed cases at admission (73% for the Vi assay and less than 50% for both O9 and Hd assays). This may have been due to the use of single urine sample collected on admission in the test. In addition, the release of S. Typhi antigens into urine was found to be intermittent and small antigens could have been destroyed by freezing and thawing. The intermittent nature of S. Typhi antigen release explains the sharp increase of assay sensitivity from 73% to 97% (for the Vi antigen) when more than one sample from the same patient were tested. A similar trend was seen with the O9 and Hd antigens.

When one considers the suitability of each antigen as a diagnostic tool for typhoid patients, the Vi antigen showed the most promise, although only 73% of culture-confirmed patients were detected with the ELISA when the baseline samples were tested. The sensitivity of the Vi antigen assay was higher than that of the O9 or Hd assays and 72% of the positive cases were strongly positive. In contrast, when the O9 and Hd antigen assays were used, less than 50% of cases were detected at admission in culture-confirmed cases. Half of the positive cases were weakly positive. Furthermore, the Vi antigen was found to be more persistent after antimicrobial therapy than either O9 or Hd antigens.

In the present study, the ELISA detected Vi antigen in 100% of the cases admitted during the first week of fever onset. It was also observed that the level of antigenuria decreased with time. The O9 and Hd antigens followed a similar time course pattern of antigenuria (decreasing by time from the fever onset date and clearance from urine) as the Vi antigen, although the proportion of cases detected was much lower than those detected with the Vi antigen.

When we used the assays to test samples from typhoid patients after antimicrobial therapy, the O9 and Hd antigens were detectable until four days after collection of the positive blood culture. However, the Vi antigen was detected 10 days after the collection of the positive blood culture. This suggests that the antigen detection assays could be used for diagnosis of typhoid patients even if they had received antimicrobial therapy, an advantage over blood culture. Accordingly, there would be no need to wait for a blood culture result. The ELISA results could be obtained on the same day as collection. In addition, the ELISA technique is easy to perform, and reagents are cheap and commercially available. Also, compared with blood culture, it could be suitable more in areas where typhoid fever is endemic and resources are limited.

The specificity of the Vi assay exceeded 90% when assessed among febrile and healthy control subjects without blood culture-confirmed typhoid fever. However, surprisingly, the specificity was only 25% when assessed among patients with
blood culture-confirmed brucellosis, despite the use of monoclonal antibodies to Vi (an improvement to the ELISA suggested by other investigators). This indicates that Brucella species may carry a Vi-like antigen, or that a Vi-like antigen is released into the urine of patients with bloodstream infections, perhaps as part of the renal component of the acute phase response. We were unable to find previous reports of Vi-like antigen from the urine of patients with brucellosis. Explanations include that brucellosis patients have never been selected as a control group when evaluating ELISAs for the diagnosis of typhoid fever. Positive reactions persisted even after experiments to address Brucella interference or cross-reactivity. This included absorption of the anti-Vi polyclonal antibody with attenuated Brucella cells which also compromised S. Typhi typhoid reactivity.

We have developed a rapid, non-invasive, and sensitive ELISA for the detection of S. Typhi antigens in urine specimens obtained from patients with typhoid fever. Testing of urine during the first week of fever onset for Vi antigen using the ELISA with a monoclonal Vi capture antibody detects most patients with typhoid fever. The assay was highly specific when evaluated with healthy and febrile control subjects with illnesses other than brucellosis. However, cross-reactivity seen among patients with brucellosis needs further investigations. In addition, typhoid patients from different age groups and different geographic areas should also be studied. Patients with a wide range of other causes of bloodstream infection should be enrolled as an additional control group for further evaluation of this assay.

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