Development of a Simple, Peripheral-Blood-Based Lateral-Flow Dipstick Assay for Accurate Detection of Patients with Enteric Fever

Iqbal Hassan Khan, M. Abu Sayeed, Nishat Sultana, Kamrul Islam, Jakia Amin, M. Omar Faruk, Umama Khan, Farhana Khanam, Edward T. Ryan, Firdausi Qadri

Incepta Pharmaceuticals Ltd, Savar, Dhaka, Bangladesh; International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh; Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts, USA; Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA; Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts, USA

Enteric fever is a systemic infection caused by typhoidal strains of Salmonella enterica and is a significant cause of mortality and morbidity in many parts of the world, especially in resource-limited areas. Unfortunately, currently available diagnostic tests for enteric fever lack sensitivity and/or specificity. No true clinically practical gold standard for diagnosing patients with enteric fever exists. Unfortunately, microbiologic culturing of blood is only 30 to 70% sensitive although 100% specific. Here, we report the development of a lateral-flow immunochromatographic dipstick assay based on the detection of Salmonella enterica serovar Typhi (S. Typhi) lipopolysaccharide (LPS)-specific IgG in lymphocyte culture secretion. We tested the assay using samples from 142 clinically suspected enteric fever patients, 28 healthy individuals residing in a zone where enteric fever is endemic, and 35 patients with other febrile illnesses. In our analysis, the dipstick detected all blood culture-confirmed S. Typhi cases (48/48) and 5 of 6 Salmonella enterica serovar Paratyphi A blood cultured-confirmed cases. The test was negative in all 35 individuals febrile with other illnesses and in all 28 healthy controls from the zone of endemicity. The test was positive in 19 of 88 individuals with suspected enteric fever but with negative blood cultures. Thus, the dipstick had a sensitivity of 98% compared to blood culture results and a specificity that ranged from 78 to 100% (95% confidence interval [CI], 70 to 100%), depending on the definition of a true negative. These results suggest that this dipstick assay can be very useful for the detection of enteric fever patients especially in regions of endemicity.
TABLE 1 Characteristics of study participants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value for the subject group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Suspected enteric fever (n = 142)</td>
</tr>
<tr>
<td>Median age (25th and 75th percentiles [yr])</td>
<td>7 (3, 11)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>No. of male subjects (%)</td>
<td>71 (50)</td>
</tr>
<tr>
<td>No. of female subjects (%)</td>
<td>71 (50)</td>
</tr>
</tbody>
</table>

Values are presented as median (25th and 75th percentiles) and number (percentage) as appropriate.

TABLE 2 Characteristics of study participants with suspected enteric fever

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value by test results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size (no.)</td>
<td>Positive blood culture and dipstick</td>
</tr>
<tr>
<td></td>
<td>53</td>
</tr>
<tr>
<td>Median age (25th and 75th percentiles [yr])</td>
<td>6 (3, 12)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>No. of male subjects (%)</td>
<td>18 (34)</td>
</tr>
<tr>
<td>No. of female subjects (%)</td>
<td>35 (66)</td>
</tr>
<tr>
<td>Duration of fever (25th and 75th percentiles [days])</td>
<td>5 (6, 20)</td>
</tr>
<tr>
<td>No. of subjects with prior use of antibiotics (%)</td>
<td>9 (17)</td>
</tr>
</tbody>
</table>
card to overlap the conjugate pad to facilitate the flow of sample from a specimen vial to the strip. To accelerate migration of the samples through the strip, we used cellulose fiber as an absorbent pad pasted on the backing card opposite the conjugate pad. All pads were cut to make the desired strip shape by using a guillotine cutter (CT300 and ZQ2000; Kin Biotech Co., China).

Separation of peripheral blood cells by erythrocyte lysis. To recover peripheral lymphocytes, we lysed erythrocytes present in 1 to 2 ml of venous blood using lysis buffer (0.13 M ammonium chloride, 1 mM potassium bicarbonate, and 0.01 mM disodium EDTA) at a 1:5 dilution and mixed the sample by gently inverting the tube (BD Falcon) three to five times. The tube was then kept at room temperature for 10 min and centrifuged at 953 × g for 5 min at 20°C. We decanted the supernatant and resuspended the pellet in 150 μl of RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 1% penicillin-streptomycin (Gibco), 1% sodium pyruvate (Gibco), and 1% L-glutamine (Gibco). We cultured the suspended cells in culture vials (North China Pharmaceuticals Co. Ltd., China) without any antigenic stimulation at 37°C without 5% CO₂ for 48 h. We then harvested the culture suspension and centrifuged it at 11,600 × g at 20°C for 5 min to collect the supernatant.

Testing the strip. The strip contained two lines on the nitrocellulose membrane: one was the test line containing MP or LPS antigen, and the other was the control line containing rabbit anti-goat IgG (Jackson ImmunoResearch). The conjugate pad contained goat anti-human IgG or goat anti-human IgA conjugated to colloidal gold. We diluted 75 μl of the lymphocyte culture supernatant with 0.02 M Tris–1% BSA–3% Tween at a 1:1 dilution in a microcentrifuge tube and dripped the strip into the tube for 15 min. The test line and/or control line would appear as a red line. The presence of both the control line and the test line indicated that the sample was positive for the test undertaken. The presence of only the control line but no test line indicated a negative result for the test.

Detection of S. Typhi LPS-specific antibodies in specimens by ELISA. We compared strip results to those obtained using an anti-S. Typhi LPS ELISA format, as previously described (10). Briefly, we coated microtiter plates (Nunc F; Nunc) with 100 μl of LPS (2.5 μg/ml) and blocked the samples with 1% BSA in phosphate (PBS). To detect antigen-specific responses, we added 100 μl of lymphocyte culture supernatant (1:2 dilution in 0.1% BSA-PBS-Tween) to coated plates, which were then incubated for 90 min at 37°C. After plates were washed with 0.05% PBS-Tween, we added rabbit anti-human IgG (1:10,000 dilution; Jackson ImmunoResearch) conjugated to horseradish peroxidase and incubated the plates for 90 min at 37°C. We then developed the plates with ortho-phenylenediamine (Sigma) in 0.1 M sodium citrate buffer and 0.1% hydrogen peroxide after the plates were washed with 0.05% PBS-Tween. We then read the plates kinetically at 450 nm for 5 min at 19-s intervals. The maximal rate of optical density (OD) change was expressed as milliabsorbance per minute. For normalization of the data, the results were divided by readings of in-house pooled convalescent-phase standard sera of blood culture-confirmed typhoid patients and multiplied by 100. Results were expressed as ELISA units (EU).

Statistical analysis. We used GraphPad Prism, version 4, and Open-Epi, version 3, for data management, analysis, and graphical presentation.

RESULTS

Characterization of colloidal gold. After making colloidal gold, we determined the size of the gold nanoparticles by differential light scatterings using a Zetasizer Nano ZS90 instrument (Malvern Instrument, Ltd.). The measurements were carried out at 25°C with a count rate of 193.7 kcps at a scattering angle of 173°. The average diameter of the prepared gold nanoparticles was 20 nm, as determined from the dynamic light scattering (DLS) spectrum (Fig. 1).

Determination of optimum pH and minimum concentration of detection antibody for conjugation. We performed aggregation testing at different pHs and with increasing amounts of goat anti-human IgG or goat anti-human IgA to produce conjugated gold. After the addition of NaCl, we measured the optical density (OD) at 520 nm, 580 nm, and 600 nm. We used the ratio of the OD at 520 nm to that at 580 nm to assess stability and the ratio of the OD at 600 nm to that at 520 nm to assess polydispersity (20–22). We found the highest stability and lowest polydispersity when colloidal gold was conjugated to both anti-human IgG and IgA at pH 8.0 (Fig. 2). We also found that a minimum of 12 μg of goat anti-human IgG or 16 μg of goat anti-human IgA was required to stabilize 1 ml of colloidal gold solution (Fig. 3).

Results of blood culture. We enrolled total 142 suspected enteric fever patients; among them, 54 patients were blood culture-confirmed enteric fever patients. Among 54 bacteremic patients, 48 were S. Typhi-positive and 6 were S. Paratyphi A-positive patients.

Results of the strip test. We tested the immunochromatographic strips detecting S. Typhi LPS-specific or MP-specific IgA or IgG in lymphocyte culture supernatant of subsets of our cohorts. The strip detecting S. Typhi LPS-specific IgG in lymphocyte culture supernatant was positive in all (n = 48) S. Typhi bacteremic patients and in 5 of 6 S. Paratyphi A bacteremic patients. We also tested the LPS-specific IgA assay using lymphocyte culture supernatant prepared from venous blood of 88 blood culture-negative patients (clinically suspected to have enteric fever), as well as from 28 healthy controls and 35 patients with other febrile illness (tuberculosis, kala-azar, Dengue, and nonsalmonella bacteremia). We found that the dipstick was positive for 19 blood culture-negative patients and negative for all healthy controls as well as for all the patients with other febrile illness (Fig. 4 and Table 3). The strip that detected S. Typhi MP-specific IgA responses showed variable intensities of test and control lines in culture-confirmed patients, and the strip detecting MP-specific IgG responses, although detecting all blood culture-confirmed patients, was also positive in patients with other febrile illnesses.

Assessing LPS-specific antibody responses in lymphocyte culture supernatant using ELISA. We next assessed IgG antibody responses using an ELISA format and lymphocyte culture supernatant collected from our different categories of patients. Among
them, 22 patients were positive by blood culture and IgG LPS-specific strip test, 9 patients were negative by blood culture and positive by strip test, and 35 patients were negative for both blood culture and strip test. We also measured LPS-IgG responses in 16 healthy individuals and 16 other febrile-illness patients. The patients who had LPS-IgG responses of ≥16 EU were positive by the strip (Fig. 5).

**Determination of sensitivity, specificity, PPV, and negative predictive value for strip test detecting S. Typhi LPS-IgG.** We calculated the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the IgG LPS-specific lateral-flow dipstick (LFD) using OpenEpi, version 3, an open source calculator for the evaluation of diagnostic tests. The test had a sensitivity of 98% compared to blood culture results and specificity, PPV, and NPV that ranged from 78 to 100% (95% CI, 68 to 100%), 73 to 100% (95% CI, 62 to 100%), and 98 to 99% (95% CI, 91 to 99), respectively, depending on the definition of a true negative (Table 4).

**DISCUSSION**

Enteric fever remains an important public health concern in many developing countries. There is a very real need for a low-tech, reliable, and affordable diagnostic test that shows high sensitivity and specificity. In our study, we have reported development of such a test. The test can be performed using 1 ml of venous blood, erythrocyte lysis buffer, a tabletop centrifuge, and a 37°C incubator without CO₂. Responses are detected visually with a simple dipstick. Although such a test cannot be used at the bedside, results with excellent precision and reliability and minimal laboratory capacity are available at 48 h. Our previous data suggest that a reading at 24 h may also be informative (1).

The current immunochromatographic assay and the previously reported ELISA-based TPTest are both based on detection of antibodies secreted ex vivo by activated lymphocytes recovered from the peripheral circulation during acute infection (1, 10, 19). These lymphocytes have been stimulated by the recent infection and require no ex vivo stimulation. Removing the plasma component of blood limits the confounding influence of preexisting circulating antibodies that reflect prior exposure. These circulating antibodies can affect assay specificity and have markedly limited the utility of plasma antibody-based assays in areas of the world where enteric fever and salmonellosis are endemic.

We evaluated our strips using specimens from patients clini-

---

**FIG 2** Optimization of pH for anti-human IgG conjugation (A) and anti-human IgA conjugation (B). Optical density (OD) ratios of values at 520 nm to 580 nm and at 600 nm to 520 nm represent stability and polydispersity, respectively.

**FIG 3** Determination of minimum amount of anti-human IgG (A) and anti-human-IgA (B) required for conjugation of 1 ml of colloidal gold solution. Optical density (OD) ratios of values at 520 nm to 580 nm and at 600 nm to 520 nm represent stability and polydispersity, respectively.
Healthy controls 28 0 28
Febrile illness other than enteric fever 35 0 35
Clinically suspected for enteric fever
Paratyphi A bacteremia 6 5 1
S Typhi bacteremia 48 48 0

Study participant group

<table>
<thead>
<tr>
<th>Study participant group</th>
<th>No. of subjects</th>
<th>Total</th>
<th>Positive strip test</th>
<th>Negative strip test</th>
</tr>
</thead>
<tbody>
<tr>
<td>S Typhi bacteremia</td>
<td>48</td>
<td>48</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>S Paratyphi A bacteremia</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Clinically suspected for enteric fever</td>
<td>88</td>
<td>19</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>Febrile illness other than enteric fever</td>
<td>35</td>
<td>0</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Healthy controls</td>
<td>28</td>
<td>0</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 3 Results of strip test detecting LPS-specific IgG

Clinically suspected to have enteric fever, as well as specimens from healthy individuals and patients with other febrile illnesses. We defined participants whose blood cultures were positive for S Typhi or S Paratyphi A as definitive cases of enteric fever. The LPS-specific IgG lymphocyte supernatant-based dipstick had a sensitivity of 98% using this definition. We defined participants with other febrile illnesses and healthy controls as definitive negatives. The LPS-specific dipstick test had a specificity of 100% using this definition. We defined participants whose blood cultures were positive for S Typhi or S Paratyphi A as definitive cases of enteric fever. The LPS-specific dipstick test had a specificity of 100% using this definition. Since there is no clinically practical gold standard for enteric fever and since microbiologic culturing of blood is known to have a sensitivity of 30 to 70% (3, 12, 13), the interpretation of the performance of the dipstick assay in individuals with suspected enteric fever but negative microbiologic culturing of blood is problematic. Presumably, a subset of these individuals actually do have enteric fever. However, we could estimate a lower specificity value: if we assume that all blood culture-negative patients indeed do not have enteric fever, the LPS-specific IgG dipstick test would still have a specificity of 87% when all negative controls are included. These results are very promising, and considering that some of the culture-negative patients probably did indeed have enteric fever, they are very likely an underestimate of true specificity.

Our assay is based on detecting LPS derived from S Typhi. Interestingly, our test also detected patients with S Paratyphi A bacteremia. Paratyphoid fever caused by S Paratyphi A accounts for up to 1 in 5 cases of enteric fever in some areas of Asia, including Bangladesh (4), and paratyphoid and typhoid fevers can be clinically indistinguishable (4, 23). The S Typhi LPS serotype is defined by the O antigen, determined by the O-specific oligonucleotide and polysaccharides associated with the LPS. S Typhi O antigens include serotypes 9 and 12, often expressed on the same organism. S Paratyphi A antigens include serotypes 1, 2, and 12. The identification of S Paratyphi A-infected patients by the dipstick assay presumably rests upon the detection of circulating lymphocytes expressing anti-serotype 12 O-antigen antibodies in these individuals.

Invasive salmonellosis is a distinct clinical entity from enteric fever that is caused by traditionally nontyphoidal strains of Salmonella enterica, especially S Typhimurium and S enterica serovar Enteritidis. Such invasive nontyphoidal salmonellosis (iNTS) is a significant cause of mortality in malnourished and immunocompromised children, especially HIV-infected individuals in sub-Saharan Africa (24). Although we did not assess our dipstick assay in patients with iNTS (who are rare in Dhaka, Bangladesh), we are encouraged to note that both S Typhimurium and S Enteritidis can express O antigen 12, suggesting that the current dipstick assay might be able to detect at least a subset of individuals with iNTS.

Our dipstick assay has a number of limitations. It is not point of care, it requires electricity (centrifuge and incubator), it requires 24 to 48 h until results are available to the clinician, and it does not discern antimicrobial susceptibility profiles. We could not collect a large volume of blood for culture, which may be a reason for the low sensitivity of the blood culture. We enrolled adult healthy controls although suspected enteric fever patients were largely children. However, the dipstick assay is extremely simple, requires only a small volume of peripheral blood and rudimentary laboratory equipment and training, can be read with the naked eye, is as sensitive as blood culture, may be more sensitive than blood culture, and is estimated to be more specific than currently available serology assays. This dipstick assay thus may be quite useful in detecting patients with enteric fever in resource-limited settings with limited facilities, identifying those who could most benefit from appropriate treatment to prevent subsequent complications of enteric fever and minimizing the use of inappropriate antimicrobial agents that...
drive drug resistance and can cause unnecessary adverse events. This assay could also assist in defining the burden of enteric fever in resource-limited regions and could assist in judging the impact of control programs.

ACKNOWLEDGMENTS

This work was supported by the ICDDR,B and grants from the National Institutes of Health, including the National Institute of Allergy and Infectious Diseases (AI100023; to E.T.R. and F.Q.), by a Fogarty International Center Training Grant in Vaccine Development and Public Health (TW005572; to M.A.S., K.I., and F.K.), by the Bill and Melinda Gates Foundation (grant number OPP50419), and also by the SIDA fund (grants 54100020 and 51060029). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

This test was developed in collaboration with a commercial company.

FUNDING INFORMATION

This work, including the efforts of Firdausi Qadri, was funded by Bill and Melinda Gates Foundation (grants TW005572; to M.A.S., K.I., and F.K.), by the Bill and Melinda Gates Foundation (grant number OPP50419), and also by the SIDA fund (grants 54100020 and 51060029). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References


