



Evaluation of a Rapid Point-of-Care Multiplex Immunochromatographic Assay for the Diagnosis of Enteric Fever

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ABSTRACT There is a critical need for an improved rapid diagnostic for enteric fever. We have previously demonstrated that serum IgA responses targeting *Salmonella enterica* serovar Typhi hemolysin E (HlyE) and lipopolysaccharide (LPS) are able to discriminate patients with acute typhoid from healthy controls in areas where enteric fever is endemic (healthy endemic controls) and from patients with other bacterial infections. We now have data demonstrating that IgA antibody responses against these antigens also work well for identifying patients with acute *S. Paratyphi A* infection. To develop a test for acute enteric fever detection, we have adapted a point-of-care immunochromatographic dual-path platform technology (DPP), which improves on the traditional lateral flow technology by using separate sample and conjugate paths and a compact, portable reader, resulting in diagnostics with higher sensitivity and multiplexing abilities. In this analysis, we have compared our standard enzyme-linked immunosorbent assay (ELISA) method to the DPP method in detecting acute phase plasma/serum anti-HlyE and anti-LPS IgA antibodies in a cohort of patients with culture-confirmed *S. Typhi* ($n = 30$) and *Paratyphi A* infection ($n = 20$), healthy endemic controls ($n = 25$), and febrile endemic controls ($n = 25$). We found that the DPP measurements highly correlated with ELISA results, and both antigens had an area under the curve (AUC) of 0.98 (sensitivity of 92%, specificity of 94%) with all controls and an AUC of 0.98 (sensitivity of 90%, specificity of 96%) with febrile endemic controls. Our results suggest that the point-of-care DPP Typhoid System has high diagnostic accuracy for the rapid detection of enteric fever and warrants further evaluation.

IMPORTANCE Enteric fever remains a significant global problem, and control programs are significantly limited by the lack of an optimal assay for identifying individuals with acute infection. This is especially critical considering the recently released World Health Organization (WHO) position paper endorsing the role of the typhoid conjugate vaccine in communities where enteric fever is endemic. A reliable diagnostic test is needed to assess and evaluate typhoid intervention strategies and determine which high-burden areas may benefit most from a vaccine intervention. Our

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collaborative team has developed and evaluated a point-of-care serodiagnostic assay based on detection of anti-HlyE and LPS IgA. Our finding of the high diagnostic accuracy of the DPP Typhoid System for the rapid detection of enteric fever has the potential to have significant public health impact by allowing for improved surveillance and for control and prevention programs in areas with limited laboratory capacity.

KEYWORDS *S. Paratyphi A*, *S. Typhi*, *Salmonella*, diagnostic, enteric fever, paratyphoid, point-of-care, typhoid

Typhoid and paratyphoid fever, collectively known as enteric fever, affect more than 14 million people globally and result in around 135,000 deaths each year (1). Enteric fever is prevalent in low-and-middle-income countries (LMICs) that lack access to clean drinking water and improved sanitation, especially in southeast Asia, south Asia, and sub-Saharan Africa (1).

A major unresolved issue in the management, prevention, and control of enteric fever is the absence of a reliable and rapid diagnostic assay. Clinical diagnosis is unreliable (2), and the current reference standard, blood culture, has several limitations, including a low sensitivity of approximately 52 to 70% (3), a several day lag between sample collection and result availability, and requirement of substantial laboratory capacity. Serum-based diagnostics such as the Widal agglutination test, and commercially available assays such as Typhidot and Tubex, while simple and rapid, offer only moderate accuracy in specificity and sensitivity (4). Detecting antibodies secreted from circulating, activated lymphocytes (TPTest) has high sensitivity and specificity for diagnosing enteric fever, but it requires moderately advanced laboratory capacity and requires 18 to 48 h to obtain results (5–7). A rapid diagnostic for enteric fever could improve medical management, reduce overdiagnosis and overuse of antityphoid antimicrobials, which has driven antimicrobial resistance, and improve disease burden estimates and surveillance in areas where enteric fever is endemic, so that informed decisions can be made surrounding vaccine introduction (5). This is especially relevant given the WHO's prequalification and endorsement of the typhoid-conjugate vaccine (TCV) (8).

There have been several high-throughput immunoscreens of the *Salmonella enterica* serovar Typhi proteome to identify promising antigens that can be used to develop a serodiagnostic assay that allows for accurate identification of patients with enteric fever (9–13). The top candidate antigens have included *S. Typhi* lipopolysaccharide (LPS), hemolysin E (HlyE), cytolethal distending toxin B (CdtB), flagellin, outer membrane protein A (OmpA), pathogenicity island effector proteins SipB and SipC, among others (9–13). All these studies have identified antibody responses to LPS and/or HlyE among the best discriminators of acute typhoid patients from healthy controls from areas where enteric fever is endemic (endemic healthy controls) and other febrile controls (9–13). In a recent analysis, we applied supervising learning methods and two independent cohorts from Bangladesh and Nepal to identify the best antigen and antibody isotype combinations to identify patients with acute typhoid fever. We found that serum IgA responses targeting *S. Typhi* hemolysin E (HlyE) and LPS are able to discriminate patients with acute typhoid illness from healthy endemic area controls as well as from patients with other bacterial infections (14). We now have data demonstrating that IgA antibody responses against these antigens also work well for identifying patients with acute *S. Paratyphi A* infection, which accounts for 10 to 50% of enteric fever infections in areas of Asia (1, 15).

To translate the serologic testing of HlyE and LPS IgA responses into a multiplex rapid test for enteric fever, we have used Chembio's patented DPP (Dual Path Platform [16, 17]), a point-of-care immunochromatographic technology. The DPP Typhoid System consists of a sample path that distributes a small volume of sample (~10 μ l of whole blood, plasma, or serum) onto an antibody detection strip containing a test line for LPS, a test line for HlyE, and a control line (Fig. 1). Results are obtained with the DPP

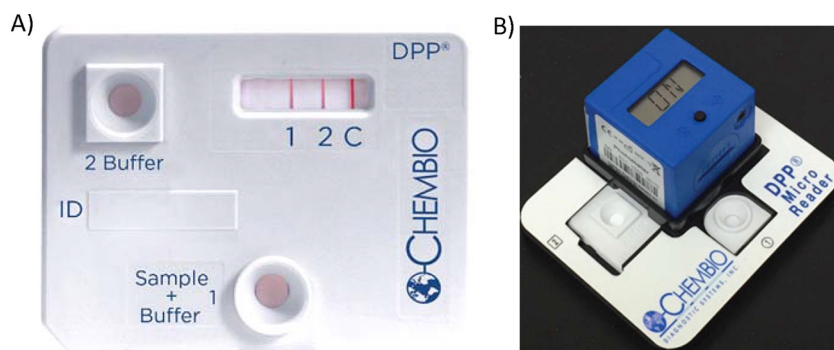


FIG 1 DPP Typhoid System. The test cassette and DPP Micro Reader with holder and typhoid test device are shown. (A and B) The DPP Typhoid System consists of a test cassette, which consist of a sample path and reagent path which intersect in the analyte detection area labeled 1 (LPS), 2 (HlyE), and C (control) (A) and the DPP Micro Reader, a portable, battery-powered instrument that displays a numerical intensity value for the test lines (B).

Micro Reader, a portable, battery-powered instrument using assay-specific algorithms to verify the presence of the control line and displays a numerical intensity value for each test line. The device has been designed to minimize human interpretation error. This multiplex system has the capability of measuring plasma IgA responses to both LPS and HlyE with high sensitivity and specificity, and its results were highly correlated with ELISA results.

RESULTS AND DISCUSSION

Characterization of anti-LPS and HlyE IgA responses. We evaluated plasma and serum IgA responses to LPS and HlyE antigens by ELISA and DPP Typhoid System using previously collected samples from three cohorts of individuals: (i) patients at the acute phase of enteric fever (day of presentation to a health facility), with blood culture-confirmed *S. Typhi* ($n = 30$) or *S. Paratyphi A* ($n = 20$); (ii) healthy controls from Bangladesh, an area where typhoid is endemic ($n = 25$); and (iii) febrile controls from Nepal with other bacteremias (i.e., *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus* spp.; $n = 25$).

We found higher IgA immunoreactivity to LPS and HlyE in *S. Typhi* and *S. Paratyphi A* cases by ELISA and DPP compared to healthy controls from areas where enteric fever is endemic (endemic healthy controls) ($P < 0.0001$) and endemic febrile controls ($P < 0.0001$) (Fig. 2 and 3, respectively).

Comparison of DPP Typhoid System measurements to reference ELISA results. The anti-LPS and HlyE IgA ELISA and DPP measurements had a high degree of linear

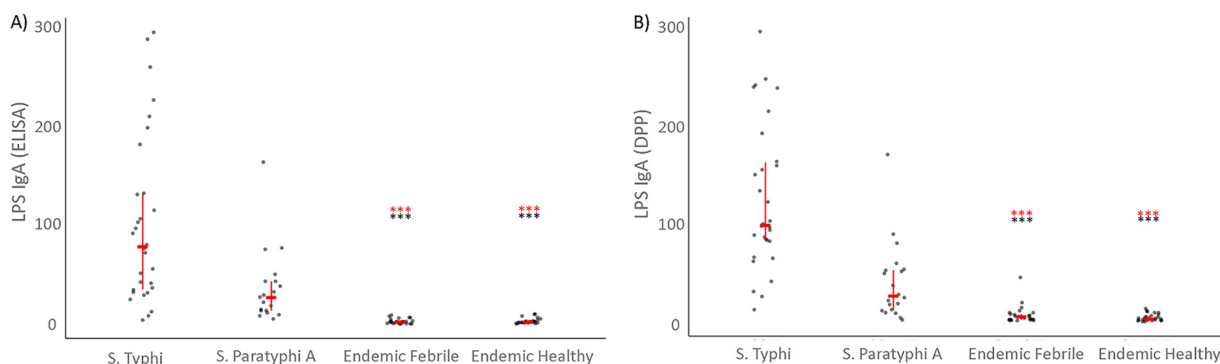


FIG 2 Characterization of anti-LPS IgA plasma responses using ELISA (A) and the DPP Typhoid System (B). Individual and median anti-LPS responses with interquartile range for patients at acute phase (day 0) of enteric fever (*S. Typhi* or *S. Paratyphi A*), healthy and febrile controls from a typhoid-endemic area (endemic healthy and endemic febrile). Differences between cases and control groups were assessed using the Mann-Whitney test. A P of <0.05 was considered significant. ***, $P < 0.0001$ (red, *S. Typhi*; black, *S. Paratyphi A*).

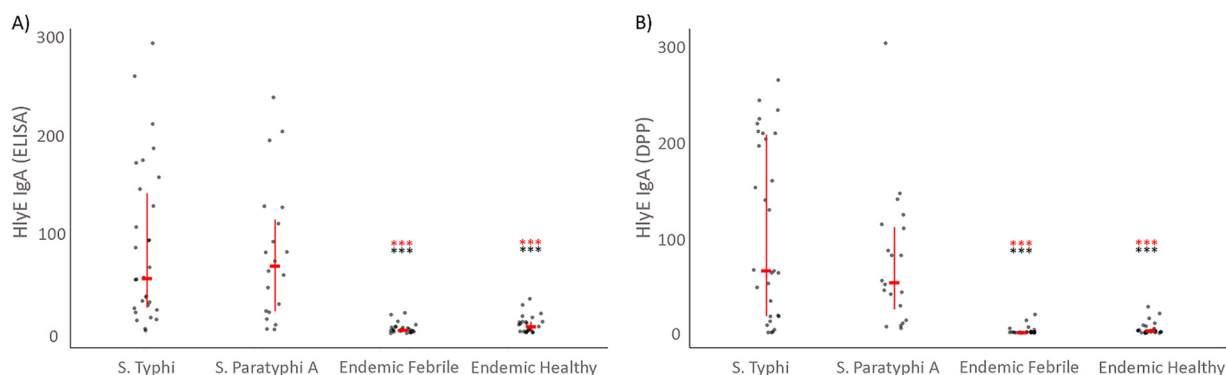


FIG 3 Characterization of anti-HlyE IgA plasma responses using ELISA (A) and DPP (B). Individual and median anti-HlyE responses with interquartile range for patients at acute phase (day 0) of enteric fever (*S. Typhi* or *S. Paratyphi A*), healthy and febrile controls from a typhoid-endemic area (endemic healthy and endemic febrile). Differences between cases and control groups were assessed using the Mann-Whitney test. A P of <0.05 was considered significant. ***, $P < 0.0001$ (red, *S. Typhi*; black, *S. Paratyphi A*).

correlation in both negative and positive serum samples, $r = 0.86$ ($P < 0.0001$) and $r = 0.82$ ($P < 0.0001$), respectively (Fig. 4). To further characterize DPP performance agreement with ELISA, we also performed a Bland-Altman plot of the log-transformed data, which demonstrated strong agreement between the two tests without significant bias (Fig. 5).

Accuracy of the DPP Typhoid System. We assessed classification accuracy of the DPP Typhoid System by receiver operating characteristic (ROC) area under the curve (AUC) (Table 1 and Fig. 6). The combined antigens (HlyE and LPS) in the DPP Typhoid System distinguished plasma from enteric fever cases from individuals presenting with other invasive bacteremias with a sensitivity of 90% and specificity of 96% (AUC, 0.98). HlyE alone had an AUC of 0.95 (sensitivity of 90%, specificity of 92%), and LPS alone had an AUC of 0.95 (sensitivity of 90%, specificity of 88%). When including all endemic controls (healthy and febrile), the DPP had an AUC of 0.98 for the combined antigens (sensitivity of 92%, specificity of 94%), AUC of 0.93 for HlyE alone (sensitivity of 90%, specificity of 84%) and AUC of 0.96 for LPS alone (sensitivity of 90%, specificity of 92%).

We also evaluated receiver operating characteristic curves for *S. Typhi* and *S. Paratyphi A* cases, independently, for each antigen (see Table S1 in the supplemental material). Use of the combined antigens (HlyE or LPS) by the DPP Typhoid System could discriminate typhoid fever from endemic febrile controls with a sensitivity of 96% and specificity of 100% (AUC, 1.00), and all endemic controls with a sensitivity of 96% and specificity of 100% (AUC, 1.00). These results were in line with our prior findings of

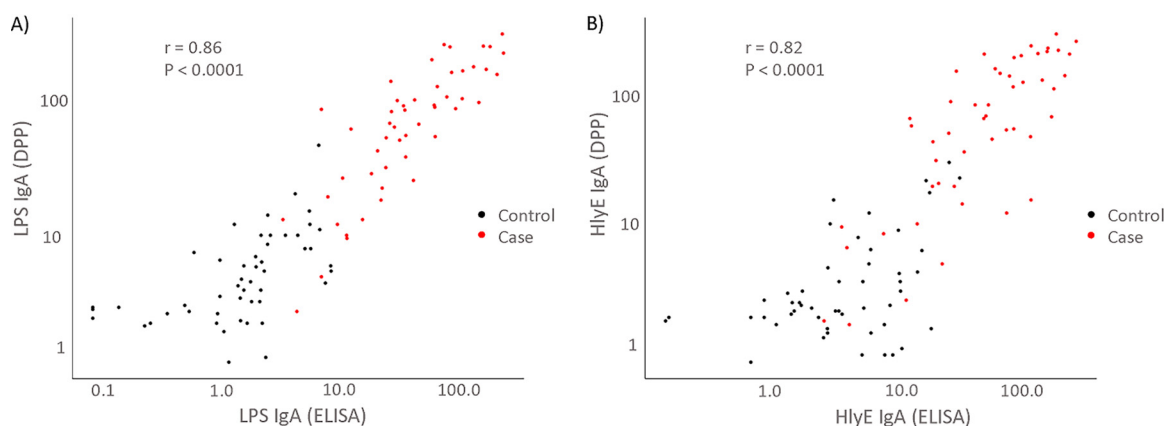


FIG 4 Correlation between ELISA and DPP Typhoid System measurements. (A and B) Plot of anti-LPS (A) and anti-HlyE (B) IgA plasma measurements by ELISA versus DPP Typhoid System of acute enteric fever cases (red, *S. Typhi* or *S. Paratyphi A*) and controls (black, endemic healthy and febrile controls). The Pearson correlation coefficient (r) is shown.

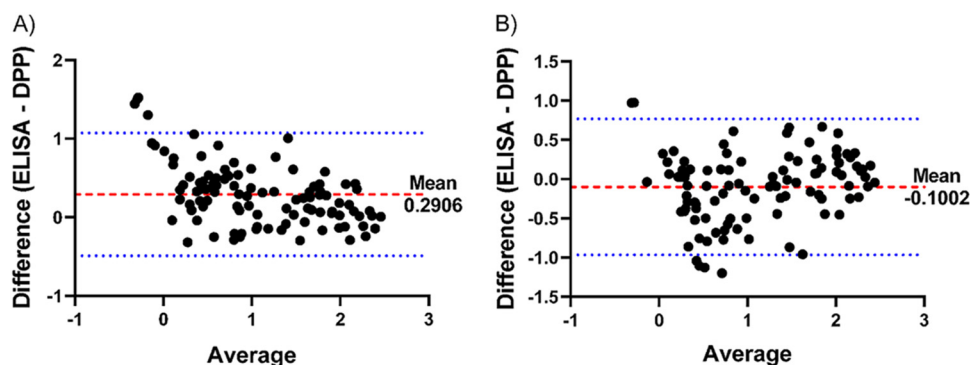


FIG 5 Bland-Altman plot of ELISA and DPP Typhoid System measurements. (A and B) Plot of log-transformed anti-LPS (A) and anti-HlyE (B) IgA plasma measurements by ELISA and DPP Typhoid System of acute enteric fever (*S. Typhi* and *S. Paratyphi* A) cases and controls (endemic healthy and febrile controls). The red dashed line indicates the mean difference between measurements. The blue dotted lines represent the 95% limits of agreement.

excellent discriminatory values for HlyE and LPS IgA responses in identifying patients with typhoid fever. The discriminatory power of anti-LPS IgA for paratyphoid A fever and controls was not as high as for typhoid fever, but the combined antigens measured by DPP could accurately distinguish samples from patients with paratyphoid fever from samples of endemic febrile controls with a sensitivity of 90% and specificity of 92% (AUC, 0.97) and from all endemic controls with a sensitivity of 90% and specificity of 88% (AUC, 0.97) (Table S1).

Conclusion. There is a critical need for a rapid, accurate diagnostic assay for enteric fever. Available rapid serodiagnostics for typhoid fever have moderate sensitivity and specificity (3), and there have been limited evaluations of rapid test diagnostics for paratyphoid fever (4). In this study, we developed and evaluated a rapid assay for the detection of patients with typhoid and paratyphoid fever, the DPP Typhoid System, based on the detection of serum/plasma IgA responses to LPS and HlyE. To evaluate the performance of the assay, we tested samples collected from enteric fever cases and controls by the DPP Typhoid System and by our reference ELISA method (14). The detection of anti-HlyE and LPS IgA in samples by DPP showed excellent agreement with results from ELISA, demonstrated high sensitivity and specificity in identifying patients with culture-confirmed enteric fever, and distinguished those individuals from controls in areas where enteric fever is endemic. These data suggest that the DPP Typhoid System may be a promising tool for diagnosing individuals with enteric fever.

Advantages of this potential point-of-care assay include its ease of handling, rapid turnaround time, minimal sample volume requirement (with potential to use 10 μ l of finger prick blood, serum, or plasma), and minimal to no requirement of laboratory capacity or training. In addition, it can identify individuals with either *S. Typhi* or *S. Paratyphi* A infection. Prior serodiagnostic assays for typhoid fever have been based on IgG and/or IgM responses to various *S. Typhi* target antigens (4). These assays have had limited sensitivity and specificity due to the high background seroprevalence of IgG

TABLE 1 Receiver operating characteristic area under the curve (AUC) for anti-HlyE and LPS IgA using DPP for distinguishing enteric fever cases (*S. Typhi* or *S. Paratyphi* A) patients from controls^a

Antigen(s)	Febrile controls			All endemic controls		
	AUC	Specificity (%) ^b	Sensitivity (%) ^c	AUC	Specificity (%)	Sensitivity (%)
HlyE	0.95 (0.90–1.00)	92	89	0.93 (0.88–0.98)	84	84
LPS	0.95 (0.90–1.00)	88	88	0.96 (0.92–1.00)	92	91
Both	0.98 (0.9–1.00)	96	90	0.98 (0.96–1.00)	98	92

^aValues for both antigens are shown in boldface type.

^bSpecificity at 90% sensitivity.

^cSensitivity at 90% specificity.

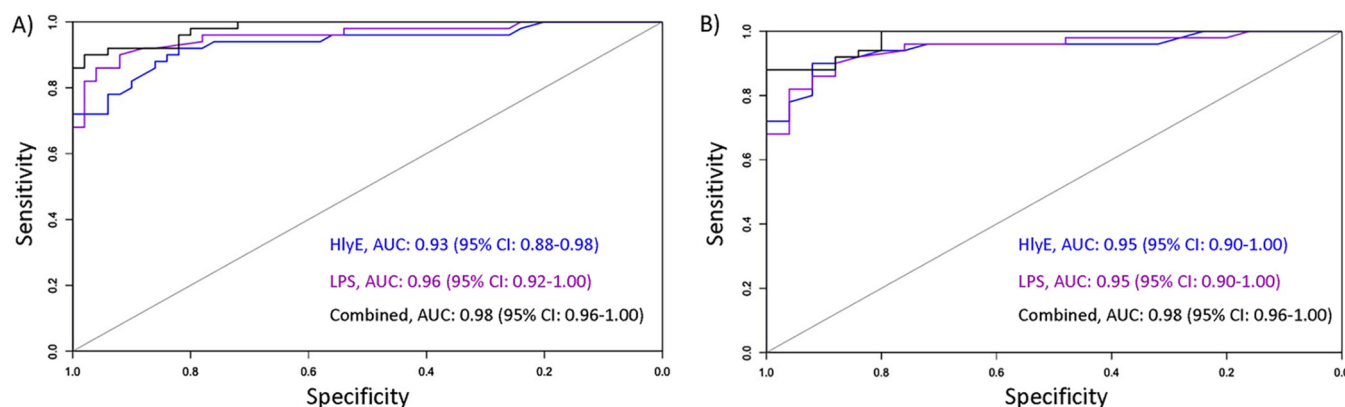


FIG 6 Receiver operating characteristic (ROC) curve for anti-LPS and HlyE IgA response using DPP for the diagnosis of acute enteric fever. ROC curve plotting the specificity versus sensitivity of distinguishing patients with acute enteric fever (*S. Typhi* or *S. Paratyphi A*) from all endemic controls (healthy and febrile controls) (A) and endemic febrile controls (B). 95% CI, 95% confidence interval.

and the cross-reactivity of IgM in areas where enteric fever is endemic (4). Measurement of IgA in the DPP Typhoid System overcomes some of these limitations due to the relative transience in plasma of antigen-specific IgA compared to IgG antibodies and improved specificity over IgM antibodies (11).

A limitation of our analysis is that the test thus far has been evaluated only with stored plasma and serum samples from a small cohort of adult cases and controls from Asia. Future studies will need to include assessment of assay performance for (i) other sample types (e.g., capillary whole blood from finger stick); (ii) other populations/settings (e.g., Africa, elsewhere in Asia); (iii) different age groups, particularly young children; (iv) different stages of illness (e.g., days of fever prior to presentation), and (v) various alternative febrile illness (e.g., dengue, chikungunya, scrub typhus, and other invasive bacteremias, particularly invasive nontyphoidal *Salmonella* [iNTS]), to evaluate for cross-reactive antibodies to HlyE and LPS. HlyE is present in human-specific *Salmonella* serovars, *S. Typhi* and *S. Paratyphi A*. Although absent in the primary *Salmonella* serovars causing iNTS (*S. Typhimurium* and *S. Enteritidis*), it can be found in other iNTS (including *S. Schwarzengrund*, *Montevideo*, *Bredeney*) (18–20) as well as several strains of *E. coli* (21, 22). We have previously demonstrated that HlyE IgA retained discriminatory value with other non-*Salmonella* Gram-negative organisms (i.e., *E. coli* and *Klebsiella*) (14). Another study of Nigerian children that included 86 *S. Typhi* and 29 iNTS culture-confirmed cases demonstrated negligible IgA responses to HlyE except for two patients with low immunoreactivity (11). This study did demonstrate some cross-reactivity to *S. Typhi* LPS in patients with iNTS (IgM > IgG > IgA) (11), which may potentially be due to conserved epitopes in the core lipopolysaccharide and lipid A regions (11). Further studies of DPP will need to be performed to investigate whether IgA responses to our selected antigens (LPS and HlyE) are able to discriminate enteric fever from iNTS.

Despite the limitations of our analysis, the excellent agreement of the DPP Typhoid System with our ELISA method, which has been tested more broadly (14), suggests that the DPP Typhoid System is a promising assay for the rapid detection of enteric fever and warrants further prospective analysis.

MATERIALS AND METHODS

Plasma/serum samples. Samples were obtained from participants with enteric fever on the day of presentation to the International Centre for Diarrheal Disease Research, Dhaka, Bangladesh (icddr), Dhaka hospital, Mirpur field site (Dhaka, Bangladesh), or Dhulikhel Hospital, a Kathmandu University Hospital (Kavrepalanchowk), Nepal, with self-reported fever of 3- to 7-day duration without an obvious focus of infection or alternate diagnosis. Bacteremia was confirmed by blood culture using Bact/Alert or Bactec 9050 automated system (BD Diagnostics) with identification of isolates by standard culture and biochemical tests (23, 24). Serum/plasma was also collected from healthy typhoid-endemic and North American controls and from North American patients presenting with an alternative febrile illness (PCR-confirmed influenza or bacteremia with *S. aureus*, *E. coli*, or *K. pneumoniae*). All samples were collected with the approval of the following Research and Ethical Review Committees and/or Institutional

Review Board for Human Subjects Research: International Centre for Diarrheal Disease Research, Dhaka, Bangladesh (icddr,b), Nepal Health Research Council (Kathmandu, Nepal), Stanford University, and Massachusetts General Hospital. Written informed consent was obtained from all individuals or their guardians prior to study participation. Additional North American healthy control samples used in this study were procured from ZeptoMetrix, Franklin, MA, USA.

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed on all the samples using S. Typhi LPS and purified HlyE as previously described (14). To compare across plates, we normalized ELISA results by calculating the ratio of each sample reading to that of a standard included on the same plate. The standard for HlyE was a chimeric monoclonal antibody to HlyE and for LPS, it was an in-house serum pool made from sera from patients with typhoid fever. The ratio was multiplied by 100 and expressed as ELISA units.

Development of chimeric HlyE MAbs. Immunizations and hybridoma development were performed by the Monoclonal Antibody Core facility of the Dana-Farber Cancer Institute, Boston, MA, as previously described (25, 26). Briefly, three mice (BALB/c, C57BL/6, and Swiss-Webster), 4 to 6 weeks old, were obtained from Charles River Laboratories (Wilmington, MA). All animals were acquired and maintained according to the guidelines of the Institutional Animal Care and Use Committee of Harvard Standing Committee. Mice were immunized at three subcutaneous sites and one intraperitoneal site with 50 μ g of purified HlyE emulsified with an equal volume of complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO). Mice were again boosted at day 14, and sera were collected. The mouse with the highest serum titer to HlyE was boosted again at day 35, and then the spleen and lymph nodes were collected, and cells were processed for fusion with SP 2/0 myeloma cells (ATCC no. CRL8-006, Rockville, MD) at a ratio of 2:1. Positive hybridomas and subclones were selected by indirect ELISA on HlyE and counterscreened with an irrelevant antigen. The sequenced variable regions of the light and heavy chains of selected hybridomas were cloned into pcDNA3.4 vector containing the human IgG1 and IgA1 heavy chain. The resulting plasmids were then transfected into Expi293F cells. Antibodies were purified from the cell culture supernatant by IgA affinity resin and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and size exclusion chromatography (SEC)-high-performance liquid chromatography (HPLC) (TSKgel G3000SWxl column; Tosho) to confirm molecular weight and purity. Sequencing, cloning, and antibody purification were performed by GenScript (Piscataway, NJ).

DPP Typhoid System. The DPP Typhoid System employs Chembio's patented Dual Path Platform (DPP) technology, which consists of a sample path and reagent path that intersect in the analyte detection area in the readout window of the test cassette that is labeled test (1, 2) and control (C) (Fig. 1). It employs an antibody conjugated (anti-human IgA) to colloidal gold dye particles and S. Typhi LPS and HlyE antigens that are bound to the membrane for capture of the antibody, if present in the sample. To initiate the test, a 10- μ l specimen is diluted with 5 drops (150 μ l) of sample buffer in a sample tube, and 100 μ l sample and buffer mixture is applied to the Sample + Buffer well (well 1) of the DPP test cassette. The specimen flows along the sample path membrane and is delivered to the test zone of the reagent strip, where specific LPS and HlyE antigens (test 1 and 2, respectively) and a control (C) (protein A) are immobilized. If the specimen contains anti-LPS anti-HlyE IgA antibodies, they bind instantly to the respective immobilized test antigens (test 1 and 2), while nonspecific antibody binds to the protein A control (C) line. Five minutes after adding the specimen, 6 drops (150 μ l) of buffer are added to the Buffer well (well 2). The buffer hydrates and releases the anti-human IgA antibody gold conjugate, which migrates to the test zone and binds to the captured IgA antibodies targeting LPS and/or HlyE in the respective test areas, producing a pink/purple line. The gold conjugate continues to migrate through the membrane, producing a pink/purple line in the control (C) area containing protein A. This procedural control serves to demonstrate that specimen and reagents have been properly applied and have migrated through the device. In the absence of antibodies against LPS or HlyE in the patient's sample, there are no pink/purple lines produced in the test 1 and 2 area.

Results were read using the DPP Micro Reader (Fig. 1) between 15 and 20 min after the addition of the sample/buffer to well 1. The DPP Micro Reader is a portable, battery-powered instrument that records the reflectance of the test strip surface and uses assay-specific algorithms to interpret the color intensity of the control and test lines. It displays a qualitative result for each analyte (reactive, nonreactive, or invalid) after approximately 3 s based on test-specific cutoff values loaded into the reader. The reader also displays numerical values of test line intensity, allowing for semiquantitative evaluation of antibody levels.

Statistical analysis. The distribution of antibody responses in culture-confirmed cases and controls was compared by Wilcoxon rank sum test. The agreement between ELISA and DPP measurements was assessed by Pearson's correlation and Bland-Altman analysis. The accuracy of ELISA and DPP were assessed by receiver operator characteristic area under the curve (ROC AUC). All analyses were performed using GraphPad Prism 8.2.0 and R software version 3.6.0 (R Project for Statistical Computing; <https://www.R-project.org/>).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TABLE S1, DOCX file, 0.1 MB.

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