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Validation of a new serology-based dipstick test for rapid diagnosis of typhoid fever

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ABSTRACT

Currently, no reliable diagnostic test is available for typhoid fever. One serology-based dipstick test, developed indigenously, was validated in this study. Preserved sera from 336 fever patients with known culture results for *Salmonella* Typhi were blindly tested by the Widal test and the new assay. Analytical sensitivities, specificities, and efficiencies for the new assay versus the Widal test were 68.8% versus 62.5%, 71.1% versus 37.1%, and 70.5% versus 43.2%, respectively ($p < 0.001$), considering *S.* Typhi–positive samples as gold standards. Thereafter, fresh sera from 102 hospital-attending children with clinical typhoid fever (including 20 confirmed nontyphoidal cases as control) were tested by both methods and analyzed statistically. The diagnostic sensitivity, specificity, and efficiency were 51.2%, 85%, and 57.8% for the new assay, and 43.9%, 65%, and 48% for the Widal test, respectively. Overall performance ability of the new assay was not better than the Widal test ($p > 0.5$). Further improvement of the new point-of-care typhoid assay is recommended before implementation in the field setup.

1. Introduction

Typhoid fever has emerged as one of the major public health problems across the globe. It is estimated that around 21.7 million cases of typhoid fever with more than 217,000 deaths occur annually in Southeast Asian countries (Crump et al., 2004). However, this estimation seems to be underreported due to poor disease surveillance and lack of reliable point-of-care diagnostic tests (Baker et al., 2010; Crump and Mintz, 2010). Accurate and rapid laboratory diagnosis of the disease is mandatory for early initiation of antibiotic treatment, thus reducing the risk of adverse outcome and mortality (Bhan et al., 2005; Bhutta and Mansurali, 1999).

Isolation of *Salmonella enterica* serovar Typhi (*S.* Typhi) in blood culture confirms the diagnosis of typhoid fever and is generally used as a gold standard for validation of new diagnostic assays. But it suffers from poor sensitivity which ranges from approximately 40% to 60% (Farooqui et al., 1991; Vallenas et al., 1985). Studies in Vietnam have reported the presence of lower numbers of the organisms (median, 1 CFU/mL; range, <0.3 to 387 CFU/mL) in peripheral blood due to frequent intake of newer generation of antibiotics by patients prior to attending hospitals (Wain et al., 1998). Bone marrow culture is more sensitive (>80%) than blood culture, and it has been documented that there are high numbers of organisms (median, 9 CFU/mL; range, 0.1 to 1580 CFU/mL) in bone marrow aspirates even with prior antimicrobial therapy, but it is rarely used due to the invasive nature of this technique (Kundu et al., 2006; Vallenas et al., 1985; Wain et al., 2001). Isolation of *S.* Typhi from stool, urine, or duodenal string cultures is another alternative test, but the results should be interpreted cautiously because it might reflect the chronic carrier state rather than acute infection (Vallenas et al., 1985).

The Widal test is the most commonly used serologic test, which measures the agglutinating antibody titers against *S.* Typhi lipopolysaccharide (LPS) "O" and flagellar "H" antigen. Test results have only 30% positive correlation with culture-confirmed typhoid fever. False positivity has been reported in fever cases other than typhoid like malaria, dengue, typhus fever, etc., which has negative influence on the reliability of the test (WHO, 2003). Determination of proper baseline cut-off titer in site-specific healthy population and examination of paired sera samples for interpretation further restricted its practical use in endemic areas (Clegg et al., 1994; Parry et al., 1999). Using molecular methods (polymerase chain reaction, DNA microarray) on direct samples might improve the case detection rate by increasing the sensitivity, but lack of standardization and expertise, and increased cost of reagents and equipment have made these methods less attractive in resource-poor settings. Therefore the search for a rapid, simple, and affordable point-of-care diagnostic test for typhoid that can be used in remote fields of endemic areas as a bedside test without the need for sophisticated equipment continues.

A number of new-generation serology-based rapid diagnostic tests for typhoid have been commercially available like Typhidot (Malaysian Bio-Diagnostics Research SDN BHD, Malaysia), TUBEX (IDL Biotech, Sollentuna, Sweden), Multi-Test Dip-S-Tics (PanBio Indx,

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Baltimore, MD, USA), whose performances have been evaluated worldwide. But none of these tests was time tested and yielded sustainable results when validated in different endemic setups (Parry et al., 2011).

A new serologic assay, based on the principles of immunochromatography as a lateral flow dip stick test (IC-LFT), was developed indigenously by SPAN Diagnostics, Surat, India. IC-LFT qualitatively detects both IgM and IgG antibodies, specific to lipopolysaccharide (LPS) and flagellin of *S.* Typhi, in human sera/plasma. The test kit was standardized and found appropriate for specific diagnosis of typhoid by the in-house test (data not published). The new assay system has undergone further external validation at the Bacteriology Division of the National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India. The result of the validation is presented in this article.

Briefly, sera samples (collected from fever cases with known culture result for *S.* Typhi), obtained from the serum bank of NICED, Kolkata, were tested in a blinded manner to determine the analytical sensitivity and specificity of the new assay. Determination of diagnostic efficiency and evaluation of performance ability of the new diagnostic test were carried out in an actual field setting by testing prospective sera samples from hospital-attending children with clinical diagnosis of typhoid fever following standard published methods (OIE, 2012).

2. Materials and methods

2.1. Determination of analytical sensitivity and specificity

Stored sera samples from NICED serum bank collected from fever patients for other studies were used for determining the analytical sensitivity and specificity in the validation assay. A number of sera samples with known *S.* Typhi culture result were selected and tested blindly by the new kit following the manufacturer's instructions by 2 separate technicians. Samples were coded for blinding by a separate person who was not involved in the study. A sample was read positive if it was found positive by both workers. All blinded study samples were also examined by the Widal test (Span Diagnostics, Surat, India), and a titer of TO ≥1:80 was considered as positive (Dutta et al., 2006). After decoding all the results of blood culture, the Widal test and the new diagnostic test were made available to a statistician for statistical analysis and comparison between two serology-based tests (new kit versus Widal) considering positive blood culture as the gold standard.

2.2. Determination of diagnostic performances of the new kit by testing prospective sera samples from clinically diagnosed typhoid fever cases in real-life situation

2.2.1. Study population

To determine the diagnostic efficiency of the new kit in a field setup, prospective sampling of blood was carried out at Dr. B. C. Roy Post Graduate Institute of Pediatric Sciences from febrile children of 2–12 years of age, attending the outpatient department of the hospital for seeking treatment from Monday through Friday from 9 am to 1 pm during the period between April 2009 to September 2010. The children, who presented with high fever (>39 °C) and other signs and symptoms suggestive of typhoid fever, were selected for blood sample collection and separation of sera irrespective of history of antibiotic intake and severity/duration of the disease. Dr. B. C. Roy Post Graduate Institute of Pediatric Sciences is the largest referral pediatric hospital in Kolkata and patients from all socioeconomic status come to the hospital either directly or after being referred from other state hospitals.

2.2.2. Sample collection and processing

Blood samples (5 mL) were collected aseptically from febrile children clinically diagnosed as having typhoid fever and were immediately inoculated (4 mL) into a Bactec Peds Plus bottle (BD Bactec System, Franklin Lakes, NJ, USA) for isolation and identification of *S.* Typhi at the bacteriology laboratory of NICED following standard microbiological techniques (WHO, 1983). Sera were separated from 1-mL blood samples and tested by commercially available Widal test kit (Span Diagnostics) and by the new assay kit. The sera samples were also tested using the Dengue IgM capture ELISA kit (Omega Diagnostics, Scotland, UK) for the diagnosis of dengue fever. Two drops of blood samples were taken on a single glass slide for a thick and thin blood film which was stained by Leishman's stain and examined microscopically for detection of malaria parasites.

2.3. Ethical consideration

The present study was reviewed and approved by the institutional ethics committee. Blood samples were collected from the febrile children after receiving informed consent from their parents or guardians.

2.4. Microbiological culture of blood

The inoculated Bactec bottles were incubated at 37 °C for 7 days in a Bactec 9120 system (Becton Dickinson, Franklin lakes, NJ, USA), and subcultures were made on MacConkey and nutrient agars (Difco, Sparks, MD, USA) when there was any alarm signal during the incubation period. Non–lactose-fermenting smooth colonies were checked for *Salmonella* by Gram stain and other biochemical tests following a standard protocol (WHO, 1983). Confirmation of identification of the isolates was done by serotyping in slide and tube agglutination test using *Salmonella* O, H, and Vi antisera (Denka Seiken, Tokyo, Japan). If there was no growth after 7 days of incubation, blood culture was read as negative.

2.5. Serologic test: the Widal test

Widal test was performed using a quantitative tube agglutination test kit (Span Diagnostics). Serum was serially diluted in physiological saline (0.85%) for titers ranging from 1:4 to 1:64. To each set of diluted serum *S.* Typhi O and H, antigen suspensions were added separately, so that the final titers became 1:40 to 1:640. The tubes were incubated overnight at 37 °C. The results were read as highest serum dilution giving a visible agglutination with a cut-off titer of TO ≥80 (Dutta et al., 2006). Widal test–positive controls were run in each batch of test.

2.6. New assay system: IC-LFT

The new test kit was used according to the manufacturer's recommendations. The kit contained antigen (*S.* Typhi LPS and flagelline) impregnated dipstick and chase buffer. A total of 200 μL of chase buffer was mixed with 10 μl of serum in a test tube. Dipstick was placed vertically inside the test tube until the mixture liquid front reached the arrow mark. The conjugate releasing pad of the dipstick was impregnated with colloidal gold conjugated to anti-human IgM and IgG antibodies. As the test sample flowed through the conjugate releasing pad due to capillary action, the anti-human IgM and/or IgG-colloidal gold conjugate formed complex with *S.* Typhi–specific IgM and/or IgG antibodies present in the sample. The complex moved on the nitrocellulose membrane and bound to the immobilized LPS and flagellin antigens. Development of one or two pinkish-red color bands on the dipstick after 20 min was considered as a positive result. Presence of an in-built immobilized control ensured the validity of the test. The test device was stable at 4–30 °C until the expiry date.