Original Article

Application of a point-of-care test for the serodiagnosis of typhoid fever in Nigeria and the need for improved diagnostics

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Abstract

Introduction: There is an urgent need for affordable point-of-care diagnostics for the differentiation of febrile illnesses and the confirmation of typhoid in endemic countries.

Methodology: Blood samples were collected from febrile patients with clinical suspicion of typhoid and screened for typhoid fever using the Widal and Typhi Dri Dot tests, while stool and blood samples were screened for *Salmonella* Typhi using the culture method as well as PCR as a confirmatory test.

Results: A high proportion of febrile patients from Lagos with clinical suspicion of typhoid fever reacted positively in a simple and rapid latex agglutination assay for typhoid fever, indicating that this illness is a common and presumably under-diagnosed health problem in this metropolis. Seropositivity was 19.2% in the rapid test compared with 22.9% in the classical Widal test. The confirmation of typhoid in these seropositive patients appeared cumbersome because of negative blood cultures and low DNA yield in molecular testing. A review of the literature revealed that in Nigeria seroprevalence rates can be high in the normal population and that pathogens other than *S.* Typhi are often isolated from the blood of seropositive febrile patients.

Conclusion: The simplicity and the relatively high specificity (97.8%) of the rapid test as determined in a study performed in Indonesia calls for a further validation of this promising test for use in Africa.

Key words: *Salmonella* Typhi; typhoid fever; diagnosis; point-of-care; Nigeria

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Introduction

Salmonella enterica serotype Typhi (*S.* Typhi), the causative agent of typhoid fever, remains a major public health problem in many parts of the world, especially in areas where clean water supplies are lacking [1,2]. In developing countries, especially in Southeast Asia and Africa, the disease is associated with high incidences of morbidity and mortality [1,3]. Worldwide, an estimated 21.6 million typhoid cases occur annually causing approximately 200,000 deaths, and the annual incidence in developing countries may be as high as 100 to 1,000 cases per 100,000 [4]. In Southeast Asia, the disease is also very common among very young children, often with high rates of complications and hospitalization [5-7]. Recent data on the prevalence of typhoid fever in Africa is lacking but in 1986 it was reported that approximately 4.36 million cases occured in an estimated population of 427 million [8].

The diagnosis of typhoid requires laboratory confirmation because other common febrile diseases may present with similar signs and symptoms. Blood culture (the gold standard) and the classical Widal agglutination test may be used for the confirmation of this disease but these methods have several limitations [2, 9-11]. The sensitivity of culture decreases with the duration of illness and culture facilities are scarce in endemic areas. The Widal test lacks both specificity and standardization. Other more recently developed serological tests such as the tube and dot rapid immunoassays have not found wide application because of the high costs and contradictory results reported in the few published

validation studies [12-16]. We developed a simple and rapid latex agglutination assay, the *S.* Typhi Dri Dot assay, for the serodiagnosis of typhoid fever [17]. This assay is well-standardized, utilizes stabilized components that do not require refrigeration, and is performed without the need for special equipment and electricity. Combined, these characteristics allow its use outside the established laboratory and in the field.

The most commonly used test for typhoid fever in Nigeria is the classical Widal test [18]. Low agglutination titres are very common in this test and as no definite cut-off value has been established, its diagnostic value is considered low and in Nigeria treatment often is commenced indifferent of the test result. To confirm the presence of *S.* Typhi in hospitalized febrile patients in Lagos, a major city in Nigeria, we applied the latex agglutination assay. This assay has a high specificity and is highly suitable for use in a hot and humid country such as Nigeria.

Methodology

Clinical specimens

Whole blood, serum and stool samples were collected from a total of 287 febrile out-patients (mean age 40.6 years, range 7 to 78 years; male to female ratio 0.65) with clinical symptoms and signs of typhoid fever. Patients were entered in the study between May 2007 and December 2008 at three health centres (the Ikorodu General Hospital, the Epe General Hospital, and the Ebute Metta Health Centre) within the Lagos metropolis.

Culture

Blood culture was performed by inoculating 2 ml of freshly collected blood into 18 ml brain heart infusion broth (Oxoid, Basingstoke, United Kingdom) supplemented with lycoid and incubating for 7 days at 37 °C. Approximately one gram of each stool sample was inoculated into Selenite F broth (Oxoid) enrichment medium and incubated at 37°C for 24 hours. Subcultures were made onto deoxycholate agar (Oxoid) and incubated at 37°C for 24 hours.

Serology

The Widal test with O antigen (febrile antigen from Chromatest, Linear Chemicals, Barcelona, Spain) was performed and interpreted according to routine laboratory procedures. A titre of $\geq$ 1:80 was considered positive.

The *S.* Typhi Dri Dot assay consists of an agglutination card containing a dot of dried, antigen-activated latex placed on the surface in the center of the card. The card is provided in a sealed moisture resistant sachet for optimal protection of the reagent during transportation and storage. The latex has a blue colour and the assay is simply performed by placing a 10 µl drop of serum next to the blue dot. The serum and the latex is then mixed using a plastic spatula provided with the card. After the latex is homogenously dispersed, the fluid is swirled by rotating the card in a near horizontal position to further mix the serum and the latex. Agglutination is readable within 30 seconds and is revealed by particulation of the latex suspension. Particulation often occurs within the first 10 to 20 seconds, and as the aggregates are usually coarse particulation is easily visible with the naked eye.

Molecular detection

DNA was prepared by proteinase K (Roche, Mannheim, Germany) digestion from 50 µl of each whole blood sample, followed by phenol-chloroform extraction and ethanol precipitation of the waterphase. Nested PCR was performed as described by Song and coworkers [19] for the amplification of a 343-bp fragment in region VI of the *fliC* flagellin gene of *S.* Typhi. The first PCR was performed by the addition of 2 µl of the extracted DNA to 25 µl PCR mixture and amplification for 25 cycles of 94°C for one minute, 56°C for 1 minute 15 seconds and 72°C for 2 minutes, followed by a primer extension of 5 minutes at 72°C using the Master Gradient Eppendorf PCR machine (Eppendorf, Hamburg, Germany). The PCR mixture consisted of standard PCR buffer (100 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% gelatin, 200 µM each of all four dNTPs, and 0.625 units of *Taq* DNA polymerase) (Roche, Mannheim, Germany) supplemented with 25 pmol of each of the 5' (TAT GCC GCT ACA TAT GAT GAG) and 3' (TTA ACG CAG TAA AGA GAG) primers (Biomers Ulm Germany). The nested PCR was performed by the addition of 2 µl of the PCR product to 20 µl standard PCR buffer supplemented with 25 pmol of each of the nested 5' (ACT GCT AAA ACC ACT ACT) and 3' (TGG AGA CTT CGG TCG GGT AG) primers and amplification for 40 cycles with the same temperature cycle program as for the first reaction. The amplification products were visualized by electrophoresis in an agarose gel stained with ethidium bromide and illuminated under UV light.

Results

Fifty-five serum samples (19.2%; 95% confidence interval [CI],15 - 24) reacted positively in the latex agglutination assay and 66 samples (23.0%; 95% CI, 18 - 28) reacted with the O antigen in the Widal test at a titre of ≥ 1:80. These 66 samples included the 55 samples that reacted in the latex agglutination assay. Of the samples that reacted in the Widal test, 39 agglutinated at a titre of 1:80 and 27 reacted at a titre of 1:160. Forty-two other samples reacted at a titre of 1:40, illustrating the poor discriminatory value of the Widal test. All blood cultures were negative for *S.* Typhi. However, *S.* Paratyphi C was isolated from the blood of one patient and *S.* Choleraesuis was isolated from the stool of another patient. Nested PCR for the detection of a segment of the *S.* Typhi *fliC* gene [19] yielded positive results for ten (3.5%; 95% CI, 2-7) blood samples, including eight samples that agglutinated in the Widal test and reacted in the latex agglutination assay. The nested PCR was negative for all Widal tests or latex agglutination assays that gave negative results. Microscopy on thick blood smears revealed the presence of malaria parasites in the blood of 53 (18.5%) patients and, of the serum samples from these patients, ten agglutinated in the Widal test and eight reacted in the latex agglutination assay.

Discussion

Our results indicate that typhoid fever is a major cause of febrile illness in the Lagos metropolis. As typhoid fever is a serious illness that requires prompt medication, the use of an accurate diagnostic test is essential. Neither culture, the Widal test nor molecular detection, was effective in recognizing the disease. All blood cultures were negative for *S.* Typhi and PCR amplification of a segment of the gene encoding the flagella was positive in only ten cases. Interpretation of agglutination in the Widal test was cumbersome as all reactive samples agglutinated at or just one titer above or below the cut-off value. The latex agglutination assay was previously validated in a study performed in South-Sulawesi, Indonesia and has a specificity of 97.9% and a sensitivity ranging from 30.8% for samples collected during the first days of illness to 84.6% for the samples collected at a later stage [17]. The high specificity of the latex agglutination assay indicates that the majority of the febrile patients with a serum sample that agglutinated in this assay indeed suffered from typhoid fever. It is possible to explain the negative culture results by the fact that most of the patients are likely to have taken antibiotics before seeking medical care at a health centre [20]; blood culture is highly sensitive to the presence of antibiotics in the sample [21]. Although in theory molecular detection of pathogen DNA by amplification should be highly sensitive, the number of organisms at any time in the blood-stream is low and the use of antibiotics may have reduced the number to below the detection level.

A number of studies have described the use of the Widal test and blood culture for the diagnosis of typhoid fever and have investigated the presence of (multi) drug resistant *S.* Typhi in Nigeria (Table 1). The prevalence of blood culture positive patients for *Salmonella* spp. ranged from 12.0% to 38.9% in febrile patients with clinical suspicion of typhoid fever for different studies. The variation could be due to differences in patient definitions and entry criteria, and in culture methods, as well as due to different epidemiological conditions. *S.* Typhi was the most frequently isolated serovar. Other *Salmonella* species that were isolated included *S.* Enteritidis, *S.* Paratyphi and *S.* Typhimurium. Seroprevalence rates measured in the Widal test were generally much higher than isolation rates and from many patients with a Widal test positive result an organism other than *Salmonella* was isolated, showing that the Widal test is highly non-specific and likely overestimates the prevalence of *Salmonella* infection. High seroprevalence rates may also be found in the normal population [36] indicating that testing a single serum sample is inadequate for the confirmation of typhoid fever. Typhoid fever in Nigeria is most common in young adults and most cases are reported in the wet season [23]. Drug-resistant *S.* Typhi is very common not only to the first-line antibiotics, but it is also increasing for other antibiotics that are not commonly used in the treatment of typhoid fever.

Health care facilities in Nigeria and in many other African countries do not have access to a well-equipped diagnostic laboratory with sufficient supplies and trained staff to perform testing for common infectious diseases [38]. Therefore, the availability of point-of-care diagnostic tests that are accurate, simple, rapid. and robust will help to improve health services and patient care. Effective point-of-care tests are needed in particular for infectious diseases that are difficult to diagnose and for which effective treatment options are available. The latex agglutination test may well be suitable for the diagnosis of typhoid fever in African countries. The agglutination cards containing the dried detection reagents are individually sealed in an airtight and

Table 1. Detection of typhoid fever patients by Widal test and blood culture in Nigeria, and (multi) drug-resistance.

City or state	Period	Description of study group	No. of patients	Percentage Widal (O antigen) test positive patients[1]	Percentage of patients with a positive blood culture	Percentage of patients with a Salmonella sp. positive blood culture	Species and prevalence (%) in population	Percent drug resistance *S.* Typhi	Percent multi drug resistance *S.* Typhi	Reference
Nsukka	1993-1994	Clinical typhoid suspects	809	13%	ND[2]	16%	*S.* Typhi (8.4%); *S.* Typhimurium (2.7%); *S.* Enteritidis (1.9%); other (2.8%)	ND	ND	[22]
Lagos metropolis	05/1997-06/1998	Clinical typhoid patients	635	69.5%	34.0%	15.9%	*S.* Typhi (10.7%); *S.* Paratyphi (2.7%); *S.* Arizona (2.5%)	100%[3]	Yes[4]	[23, 24]
Lagos	5/2000-6/2006	Pyrexia of unknown origin	103	ND	69.0%	38.9%	*S.* Typhi (38.9%)	100%[5]	61.0%[6]	[25]
Lagos metropolis	10/2004-9/2005	Febrile patients	235	ND	19.1%	17.9% *(17.0%)*[7]	*S.* Typhi (8.1%; *1.0%)*[7]; *S.* Paratyphi (3.0%; *2.0%*); *S.* Enteritidis (3.8%; *8.9%*); *S.* Arizona (3.0%; *5.4%*)	100%	80%[8]	[26]
Zaria	12/2007-6/2008	Clinical typhoid patients	713	ND	ND	14.3%	*S.* Typhi (10.0%); *S.* Paratyphi A (2.1%); *S.* Paratyphi B (2.2%)	9.9-90.1%[9]	Yes	[27]
Oyo state	2003	Clinical typhoid suspects	100	48%	ND	ND	ND	ND	ND	[28]
Akwa Ibom state	ND	Clinical typhoid patients	100	55.0%	39.0%	12.0%	*S.* Typhi (10.0%); *S.* Paratyphi (2.0%)	Yes	Yes[10]	[29]
Benue state	11/2006-6/2007	Febrile patients	1479	57.9% (26.3%)[11]	ND	ND	ND	ND	ND	[30]
Ibadan	2004-2005	Febrile poultry farm workers	991	ND	ND	3.9%	*S.* Typhi (0.7%); *S.* Enteritidis (1.3%); *S.* Dublin (0.7%); *S.* Typhimurium (0.7%); other (0.5%)	14%[12]	No	[31]
Ibadan	2004	HIV seropositive patients	108	ND	11.1%	8.3%	*S.* Typhi (4.6%); *S.* Paratyphi A (0.9%); *S.* Paratyphi B (2.7%)	ND	ND	[32]
Owerri	2005	Malaria patients	125	ND	35.2%	20%	*S.* Typhi (20%)	ND	ND	[33]
Ile-Ife	1980-1987	Sepsis	920	ND	25.3%	1.5%	*S.* Typhi (1.5%)	100[13]	ND	[34]
Ile-Ife	02/1994-01/1995	Neonates at risk of sepsis	107	ND	55.0%	1.9%	ND	ND	ND	[35]
Sagamu, Lagos and Ibadan	11/2004-5/2005	Blood bank donors	200	53%	ND	ND	ND	ND	ND	[36]
Ile-Ife	1989	Healthy school kids	408	ND	5.2%	2.0%	ND	ND	ND	[37]

1 titre ≥ 1:160 2 ND, not done/reported 3 specifically resistant to first-line antibiotics (chloramphenicol, ampicillin and cotrimoxacin) but not resistant to ofloxacin or ciprofloxacin 4 all isolates showed resistance to two or more antibiotics 5 resistant to two or more antibiotics 6 resistant to four or more antibiotics 7 results for blood samples with values for stool samples in italics 8 resistant to three or more antibiotics 9 highest resistance values observed for antibiotics not commonly used in the treatment of S. Typhi 10 resistant to three 3 antibiotics (cefotaxime, cotrimoxazole, penicillin) 11 positive for S. Typhi agglutination with percentage positive for S. Paratyphi agglutination in slide agglutination test in parentheses 12 resistant to streptomycin only 13 resistant to ampicillin and penicillin G but sensitive to first-line antibiotics

moisture resistant sachet and may be stored at ambient temperatures for at least two years without the need for refrigeration. Therefore, optimal use is made of the reagent and no excess reagent is lost or exposed to adverse conditions when testing a single patient. Apart from a pipet to apply the serum, no other equipment is required and there is no need for electricity, or special training to perform the test. However, while the evaluation study performed in Indonesia has demonstrated favourable assay characteristics in terms of sensitivity and specificity, further validation is needed to confirm the diagnostic test accuracy for use in countries in Africa. Patient characteristics and epidemiological conditions that can influence accuracy may differ from those in Indonesia. The diagnostic yield of culture is limited and in this study just ten cases that were seropositive in the latex agglutination assay could be confirmed by PCR amplification. However, PCR is not an accepted standard test for typhoid fever diagnosis and therefore further studies are needed to validate the rapid test.

The diagnosis of typhoid fever could be further improved with the development of a better standard test. Blood culture is most effective during the early stages of infection when the pathogen is still circulating in the vascular system and possibly could be improved by the use of automatic culture systems and the addition of resins to absorb antibiotics present in the sample. The culture of bone marrow is more sensitive than blood culture but this method may be used in sophisticated hospitals only, as it is painful for the patient and hence rarely applied [39-41]. Automatic culture systems were not available for use in the present study and the collection of a bone marrow sample was not possible. It is possible the yield of culture could be improved by altering the culture medium. A systematic investigation of the metabolic requirements of *Coxiella burnetti* has allowed the development of a cell-free culture medium for this fastidious pathogen [42]. Metabolomics may also help to identify volatile metabolites that then may be used as biomarkers in breath analysis [43,44]; typhoid patients are known to produce a typical smell of freshly baked brown bread [45]. The diagnostic sensitivity of PCR may be increased by simultaneously testing blood, stool and urine samples [46]. Hatta and Smits showed that the diagnostic yield of blood culture, PCR on blood, PCR on stool, and PCR on urine are 57.1%, 78.2%, 39.5% and 65.6% respectively and that the total diagnostic yield of PCR when applied on three specimens is 95.7%. Real-time PCR and multiplex PCR may have even higher sensitivities [47,48]. It is also possible that the yield of PCR could be improved by using automatic DNA extraction systems.

Essentially all serological tests for typhoid are based on the detection of antibodies to lipopolysaccharide (LPS) antigens (O9 and O12) [12-15, 17, 49]. As shown in the lateral flow assay for the detection of *S.* Typhi LPS specific antibodies, antibodies first start to develop at a time when the pathogen is already disappearing from the blood-stream [50]. The natural course and magnitude of the immune response seems to limit the sensitivity of serological testing for typhoid. Proteomic approaches could therefore be useful in identifying novel specific antigens that elicit antibodies at a very early stage of infection and new technologies such as bead-based array technology could help to improve serological testing for typhoid [51].

While further validation of existing point-of-care tests for typhoid and the development of improved diagnostic tools have high priority in the improvement of patient detection and management, control programs composed of providing both clean drinking water supplies and adequate community information to prevent infection, as well as offering vaccination to high-risk groups should also be prioritized [52]. Much attention is given to the so-called group of neglected tropical diseases, but typhoid fever remains one of the most important bacterial infections in developing countries and is thus deserving of equal attention and should be regarded and prioritized as a neglected tropical infectious disease [37].

Our results indicate that typhoid is an important infectious disease in out-patients presenting at health facilities in Lagos and that the introduction of a well-validated point-of-care test is urgently needed.

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