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Review Article

Others Section

Typhoid Diagnostics: Looking Beneath the Surface

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ABSTRACT

Typhoid fever is a systemic life-threatening infection caused by *S. typhi*, which specifically infects humans. It is a major public health concern in the developing world. The clinical symptoms of typhoid often overlap with other febrile infections, affecting timely and accurate diagnosis. The current gold standard, blood or bone marrow culture is used for definite diagnosis, however its utility is limited due to the requirement of dedicated culture facilities, technical expertise and prior antibiotic use. The existing serological tests demonstrate variable and suboptimal sensitivity and specificity, because of cross-reactivity exhibited by the conserved, somatic antigens of Lipopolysaccharides (LPS) and terminal regions of flagellin. The lack of accurate detection further leads to imprudent antibiotic use, causing complications and morbidity, and even contributes to drug resistance. This emphasises the need for superior tests for efficient typhoid diagnosis. The need has propelled the pursuit of infection-specific markers using high-yield approaches like proteomics, metabolomics, transcriptomics and potential markers have been enumerated. Bio-marker discovery serves as a start point in diagnostic development and the subsequent assessment of the diagnostic tests must be governed by pre-defined metrics to determine their accuracy and field-feasibility. The evaluation of sensitivity and specificity in endemic regions is particularly important. In the present review, we discuss the existing diagnostic modalities; their challenges and the novel approaches being utilised for the discovery of specific biomarkers. We also review the new diagnostic tests under development while also discussing the important metrics to be considered during development of improved tests.

Keywords: Biomarkers, Proteomics, Sensitivity, Specificity, *S. typhi*

INTRODUCTION

Typhoid fever is a febrile illness caused by *Salmonella enterica* serovar Typhi (*S. typhi*). The global incidence estimates vary between 11.9 million and 26.9 million cases per year, and mortality estimates range between 129000 and 161000 annually [1]. The prevalence of typhoid is high in developing nations with poor hygiene standards [2,3]. It mainly afflicts children, and vaccines based on Vi and Ty21 are recommended for routine immunisation in endemic countries [2,4]. Typhoid manifests 1-2 weeks post-ingestion of an infectious dose of *S. typhi*, with highly non-specific clinical features, making differential diagnosis difficult [5]. *S. typhi* causes systemic infection by evading host's immune system and penetrating deeper tissue [6-8]. Despite its high disease burden, accurate diagnosis of typhoid is still challenging. The gold standard-blood and bone marrow aspirate culture requires adequate infrastructure and trained personnel, unavailable in resource-poor regions. Although, rapid diagnostic tests like TUBEX and Typhidot have shown improved diagnostic accuracy over the routinely used Widal test and can potentially be employed as Point-Of-Care Tests (POCTs), however they lack optimal sensitivity and specificity [9,10], since, most of them employ detection of antibodies to surface antigens that are conserved among *Enterobacteriaceae* [11], and against which background antibody titre is high in the endemic regions. This diagnostic gap leads to imprecise disease burden estimates, misdiagnosis, and drives imprudent antibiotic use in endemic regions [12], thus, resulting in antimicrobial resistance [13,14]. Therefore, there is an urgent need for superior typhoid diagnostics, and to establish a well-defined metric system for gauging the operational features of new tests for their systemic validation [15]. The major challenge while developing improved diagnostics is either the low bacterial count or the lack of a specific biomarker [16]. Therefore, Rapid Diagnostic Tests (RDTs) employing more specific antigens are needed [17].

Recently, techniques like transcriptomics, metabolomics, and proteomics have been exploited to identify biomarkers unique to *S. typhi* bacteremia patients. The candidates identified through techniques like proteomics have demonstrated good diagnostic potential in some recent studies [18]. Additionally, some novel methods are also being explored, such as IgA detection based on a new format [19,20].

In the present review, we briefly discuss the current typhoid diagnostics and highlight the approaches for the identification of specific biomarkers. We also outline the diagnostic metrics essential for the development and evaluation of new assays, and the new tests for typhoid diagnosis.

CURRENT DIAGNOSTIC PLATFORMS

Pathogen detection: *S. typhi* detection via blood or marrow culturing has been the mainstay of clinical diagnosis. The turnover period of blood culture is 2-4 days. Fully automated blood culture systems are popular in the developed countries. However, these are not viable for the resource poor regions. Although, highly specific, blood culturing has a low and variable sensitivity (40-60%) due to the transient bacterial presence in blood and previous antibiotic treatment [21,22]. Positive detection requires higher blood volume (5-10 mL). Bone marrow culture with a sensitivity of 80-95% is valuable in cases where there is prior antimicrobial treatment, or in long-standing illness. However, it is invasive and demands expertise and equipment, hindering its regular use.

Nucleic acid-based detection: Amplification of *S. typhi*-specific genes using Polymerase Chain Reaction (PCR) or real-time PCR approach is currently being explored. While Massi MN et al., amplified *fliC* gene from all culture positive and negative blood samples with a higher copy number in culture positives *vs.* culture negatives [23]. A higher copy number contradicts the microbiological results, indicating the presence of more dead than live bacteria in patients. The most frequently assessed molecular targets include; the Hd Flagella gene, *fli*C-d [24], the Vi capsular gene *viaB* [25], the tyvelose epimerase gene (tyv), groEL gene [26], of which the *fli*C-d and the Vi locus are non-specific [27]. Prabagaran SR et al., have demonstrated the nested-multiplex PCR technique to detect

fliC and *viaB* operon as an alternative detection method [28]. The clinical efficacy of this costly approach is uncertain due to its low sensitivity (38-42%) [29].

Surface-antigen detection: Body fluids such as urine show intermittent presence of bacterial antigens and have been utilised for detection. Antigenic cross-reactivity is overcome by using Monoclonal Antibodies (MAbs) specific to surface antigens, and sensitivities up to 95% have been reported [30-32]. In an ELISA based study, Vi antigen showed 73% sensitivity which increased to 97% with sequential urine collection, although specificity remained suboptimal. O9 and Hd antigens were positive in less than 50% of culture-confirmed cases. The sensitivity was found to be highest in the first week [33]. Studies using surface antigens as markers have shown inconsistent results and are not in clinical use [34].

Antibody detection: Widal test involves the detection of agglutinating antibodies against LPS(O) and flagellar (H) antigens in suspected patients and is extensively used in endemic regions [35]. The test requires paired serum taken 10 days apart, and a positive outcome is established by a four-fold increase in antibody titre, which often occurs before the clinical onset of Typhoid. This makes it challenging to demonstrate the rise each time for positive diagnosis [36]. A single agglutination test has inadequate sensitivity and specificity [37]. The RDTs aim at reliable, rapid, uncomplicated and point-of-care diagnosis, and mainly exploit the O and H antigens of *S. typhi*, in different formats. Inhibition assay format based TUBEX®TF test detects IgM against an immuno-dominant and rare O9-antigen [37]. The ability of serum antibodies to inhibit the binding between the O9-specific mAb-coated coloured latex particles and LPS antigen coated magnetic beads further improves its specificity [38]. Another immuno-dot based method, Typhidot detects specific IgG and IgM against a 50 kDa Outer Membrane Protein (OMP) [39]. Its modification, Typhidot-M prevents masking of IgM by IgG antibodies during re-infection, through inactivation of IgG, thus improving accuracy [40,41]. The operational and technical features of commonly used commercial RDTs are summarised in [Table/Fig-1] [6,9,35,40-56].

STRATEGIES FOR IMPROVING TYPHOID DIAGNOSTICS

Discovery of novel biomarkers: The specificity of the antigens used significantly affects the performance of a test, especially in endemic regions with a high background antibody titre to conserv surface antigens. Therefore, identifying specific, sero-reactive biomarkers would enable more accurate detection [57]. *S. typhi* expresses many factors to counter host defenses or gaining replicative niche. They may exhibit good diagnostic potential. The next-generation approaches for typhoid bio-marker identification are described below. These are also summarised in [Table/Fig-2] [18,58-73].

Immuno-proteomics: In Vivo-Induced-Antigen Technology (IVIAT): Researchers have looked into the immunogenic antigens and *S. typhi*-specific antibody responses in typhoid patients using immuno-proteomics [19,20]. Harris JB et al., identified 35 immunogenic *S. typhi* antigens expressed specifically during infection using IVIAT [58]. A protein library of *S. typhi* was screened to identify immunoreactive clones using convalescent-phase sera, previously adsorbed against in vitro-grown *S. typhi* and *E. coli.* PagC, TcfB, STY1648 and STY3683 proteins were reported as *S. typhi*-specific. In a similar study, YncE was identified as a potential biomarker for carriers [74]. Since, IVIAT does not consider non-protein antigens and the possibility of cross-reactivity of homologous antigens, it warrants additional evaluation to assess the true role of such antigens in diagnostics.

Liquid Chromatography-Mass Spectrometry (LC-MS)-Based Proteomics (LC-MS): By utilising LC-MS based proteomics strategy, a group has characterised the *S. typhi* proteome under the conditions that mimic infective stage, with the goal to identify putative *S. typhi* specific virulence factors. Further, the comparison between the proteomes of *S. typhi* and *S. typhimurium* revealed that proteins like CdtB, HlyE, and gene products of t0142, t1108, t1109, t1476, and t1602, were unique to *S. typhi* [59].

Immunoaffinity Proteomics-based Technology (IPT): This involves direct screening of *S. typhi* lysate against columns charged with IgG, IgM, or IgA fractions, obtained from the patient's blood [60], using IPT this group identified 57 *S. typhi* antigens including known immunogenic proteins (PagC, HlyE, OmpA, and GroEL) and several *S. typhi* and *S. paratyphi* A specific proteins (HlyE, CdtB, PltA, and STY1364). Isotype characterisation of antibody responses is possible, thereby, determining the actual stage of infection during which antigenic presentation occurs, like in the case of *S. typhi*, which has both mucosal and systemic phases of infection. Proteins including HlyE, GroEL, and DnaK were identified by the IgM fraction (acute stage), while PagC, HlyE, CdtB, PltA, PhoN, and GroEL, reacted with either acute- or convalescent-phase IgG antibody fraction. Some proteins like the PhoP-regulated protein, PhoN and bacterioferritin reacted only with convalescent-phase IgG, thus indicative of a maturing immune response.

Microarray: Liang L et al., carried out microarray immune-profiling of *S. typhi* proteome by probing it with sera of acute typhoid patients and controls, and identified potential IgM and IgG candidate antigens, which were differentially reactive in infected patients vs healthy controls [61]. Amongst other identified antigens, two serodiagnostic candidates; haemolysin E (hlyE) and putative toxin-like protein (CdtB) were also identified in a previous study [60].

SILAC: One group identified several serovar-specific proteins by quantitatively comparing the proteomes of *S. typhi* and *S. typhimurium* using Stable Isotope Labeling with Amino acids in Cell culture (SILAC) technology [75]. *rfbE (*CDP-tyvelose-2-epimerase), and *rfbV* (Putative glycosyl transferase) were a few *S. typhi* specific antigens amongst the total of 15 antigens, which warrant further assessment.

Antibody in Lymphocyte Supernatant (ALS) assay: *S. typhi* interacts with the intestinal mucosa and induces mucosal immune response. Activated mucosal lymphocytes migrate from intestinal tissue and circulate within peripheral blood before rehoming to mucosa [76]. This response peaks 1-2 weeks post-infection, and could be measured using Peripheral Blood Mononuclear Cells (PBMCs) in an ASC assay [77] or in PBMC supernatants (ALS assay) [62]. By probing *S. typhi* protein arrays with ALS of infected patients, researchers identified immuno-reactive antigens that could discriminate between healthy, acute and non-typhoidal cases [78]. Among these antigens, Haemolysin E (HlyE), a pore forming toxin, showed highest immunoreactivity. Measuring ALS response is advantageous over serum for the diagnosis of acute infection, especially in endemic regions [79].

Metabolomics: This approach involves identification of host metabolites in response to infection [63,80]. It offers a holistic snapshot of infection, as it considers the host-pathogen interactions. By utilising two-dimensional gas chromatography with time-of-flight mass spectrometry (GC/MS), on plasma samples from typhoid, paratyphoid patients, and controls, one group has identified serovar-specific metabolic biomarkers [64]. Recently, they also identified reproducible metabolite profiles, significantly discriminating *S. typhi*-culture-positive individuals from those with alternative febrile diseases [65]. Although, it has limitations in terms of cost-effectiveness and portability, a unique combination of identified markers could be used for developing a sensitive and field deployable point-of-care test, requiring small volumes of blood.

Transcriptomics: cDNA Microarray and Selective Capture of Transcribed Sequences (SCOTS): SCOTS is a PCR hybridisation

Name of test	Typhidot	Typhidot-M	Tubex-TF	Entero-check-WB	Multi-Test-Dip-S-Ticks	Typhi-Rapid IgM & IgG IgM (Combo)	LifeAssay Testit	SD Bioline	Widal	Mega Salmonella	PanBio
Manufacturer	(Malaysian Biodiagnostic Research, Malaysia)	(Malaysian Biodiagnostic Research, Malaysia)	(IDL Biotech, Sollentuna, Sweden)	(Zephyr Biomedicals, Goa, India)	(PanBio Indx, Inc., Baltimore, MD, USA)	(CTK Biotech, Inc., San Diego, CA, USA)	(Life Assay Diagnostics (Pty), Africa)	(Standard Diagnostics Inc., Korea)		(Mega Diagnostics, CA, USA)	(PanBio Indx, Inc., Baltimore, MD, USA)
TECHNICAL FEATURES											
Assay format	**Dot Blot /Immunodot**		**Inhibition magnetic binding assay**	**Dipstick**		**Immuno-chromatographic Lateral-flow assay (ICT LFA)**			**Slide or Tube Assay**	**ELISA**	
Target of detection (antibody)	IgM & IgG	IgM (after IgG removal)	IgM	IgM	IgM & IgG	IgM	IgM	IgG/IgM	IgM & IgG	IgM & IgG	IgM& IgG
Antigen	50 kDa OMP of S. Typhi		O9 antigen (a-D tyvelose) of S. Typhi	LPS		50 kDa OMP	LPS	Undefined antigen	Flagellar (H) & LPS (O)	Undisclosed antigen	LPS
Sensitivity[a]	67-98%	47-98%	56-100%	89%	89%	89-100%	59%	69%	Variable	91%;	78%
Specificity[a]	58-100%	63-93%	58-100%	97%	53%	85-89%	98%	79%,		49%,	80%,
OPERATIONAL FEATURES											
Turnaround time	2.5-3 hours		10 minutes	15-30 minutes	90 minutes	15-30 minutes			2 hours-overnight (classical); 5 minutes (rapid centrifugation)	2-3 hours	
Sample volume (µL)	2.5		30-40 (one drop)	30-40	10	5-10			300 (two dilutions)	5-10	
Infrastructure required	none		none	none		none			Centrifuge, incubator	Incubator, Microplate ELISA reader	
Storage temperature	2-8°C		4°C to 28°C	4°C to 28°C		4°C to 28°C			2-8°C	2-8°C	
Cost[b] ($)	5		4	4	10	4.9			0.5	4.9	
Remarks	1. High IgG concentration may give false negative for IgM 2. Difficulty in differentiation between acute and convalescent cases. 3. Qualitative assay; Quantitative determination of antibodies levels not possible		1. False positives in case of Salmonella Enteritidis infection-common O9 antigen, may lead to improper treatment. 2. Subjective interpretation of color reactions. 3. Haemolysis may result in difficulty in interpretation	1. Specific IgG may compete with the IgM for sites and may result in a false negative. 2. High titer Rheumatoid factor may result in a false positive reaction 3. Cross reactivity may be observed with S. paratyphi infection		1. Qualitative or semi-quantitative results			1. Suboptimal specificity-Cross-reactive O&H Ags. 2. Lack of standardisation of reagents 3. Subjective result interpretation	1. Requires laboratory infrastructure & skilled personnel. 2. Processing time 3-4 h	
References	[40,41,83-86]		[6,9,40,41,83,85,87]	[87,88]		[40,68,69,89,90]			[35,91-94]	[40,95]	

[Table/Fig-1]: Technical and operational features of commercial Rapid Diagnostic Tests for Typhoid diagnosis [6,9,35,40,41,68,69,83-95].
[a]Denotes a range of sensitivity and specificity values from different studies with a range of sample sizes and methodologies
[b]The cost of tests have been determined from the following references: [40,87]

method of capturing bacterial genes expressed in vivo under specific growth conditions by subtractive hybridisation and PCR amplification with tagged primers. Using DNA microarray and SCOT-cDNA hybridisation approach, a group has obtained high quality global transcript profile of intracellular *S. typhi* from infected human macrophages [66]. Similarly, one group has identified *S. typhi* mRNA expressed in the blood of infected patients in order to provide insight into its pathogenesis and also identify novel host-pathogen interactions [67].

mRNA profiling and microarray: A group has investigated host responses to typhoid fever by transcriptional profiling of peripheral blood of naturally infected patients, using microarray analysis, and found that typhoid fever stimulates a distinct and reproducible peripheral blood signature that is changes during treatment, convalescence and returns to normal as found in case of uninfected controls [81]. Using similar technique another group has identified host transcriptional profiles of *S. typhi* infected young children, to provide a detailed genomic component of immunological response, and propose insights for better diagnosis and treatment [82]. The unique transcriptomic signature has the potential to form the basis of future RDTs [83].

Genomics: Advances in the field of genomics have enabled *S. typhi* genome sequencing and helped in the improved understanding of the pathogen and identification of specific gene targets. However, commonly used target genes like H antigen flagellar gene (fliC-d) and Vi capsular antigen gene (viaB) etc., are not specific to *S. typhi* only, thus diagnostic markers that can detect pathogens at single-gene target resolution could lead to a simpler, cost-effective, and more functional DNA-based detection method due to less primers requirement for target detection. A group has identified new DNA markers specific for *S. typhi* by using the genome database and nucleic acid sequence alignment tools (BLASTn). The diagnostic sensitivities and specificities of the primers designed for amplifying specific gene sequences was validated using a panel of confirmed bacteria *S. typhi*, non-Typhi *Salmonella*, and non-*Salmonella* clinical isolates [84].

Category	Technique	Methodology	Potential biomarkers/ gene targets/ metabolic markers	Biomarker; method; (Current development/ evaluation status)
Proteomics	*In vivo-induced antigen technology (IVIAT)*	Screening of S. typhi protein library expressed in Escherichia coli with convalescent-phase sera to identify immuno-reactive clones	PagC, TcfB, and STY0860 and STY3683 (45)	**CdtB, yajI, pspB, pilL, ybgF: Detection of antigen specific IgM in plasma from patients with confirmed typhoid (93) HlyE (t1477); Detection of specific IgA, IgG, IgM by ELISA(18) HlyE; Detection of salivary anti-HlyE IgA (71) HlyE: Detection of specific antibodies by Immunoblot and ELISA(72,94) (Clinical Evaluation)**
	liquid chromatography-mass spectrometry (LC-MS)-based proteomics strategy	Identification of S. typhi-specific virulence factors expressed in conditions that mimic the infective state by comparative proteomics analysis with S. typhimurium	CdtB, HlyE, and gene products of t0142, t1108, t1109, t1476, and t1602(95)	
	Immuno-affinity Proteomics-based Technology (IPT)	Capture of S. typhi proteins using columns charged with antibodies (IgM, IgG & IgA) from infected patients followed by identification of immunogenic proteins by mass spectrometry	HlyE, CdtB, PltA, and STY1364 (47)	
	Stable Isotope Labelling with Amino acids in Cell culture (SILAC) technology	Quantitative comparison of the proteomes of S. typhi and S. typhimurium for identification of serovar specific biomarkers	rfbE (CDP-tyvelose-2-epimerase), and rfbV (Putative glycosyl transferase) (49)	
	Antibody in lymphocyte supernatant [ALS] assay	Identification of S. typhi specific antigens by probing S. typhi protein arrays with ALS of patients with confirmed S. typhi bacteraemia	HlyE, CdtB, PhoN, SthD, SthA, BcfA, HpcR, Prc, EutN, and OmpS2 (53)	
	Immune profiling	Identification of S. typhi specific antigens by probing its proteome microarray with sera from infected patients	Hemolysin E (hlyE, t1477) & putative toxin-like protein (cdtB, t1111), ssaP(t1285), pilL(t4239), pspB (t1594), yajI (t2449), ybgF(t2126) (48,72)	
Transcriptomics	cDNA Microarray and Selective capture of transcribed sequences (SCOTS) to identify high quality transcript profiles from intracellular bacteria	SCOTS to identify global genes expression of S. typhi during infection (human macrophages) SCOTS and microarray hybridisation to identify S. typhi transcripts expressed in the blood of patients infected with S. typhi in Bangladesh	up regulated genes: STY1482, STY1353, STY1361-STY1367, STY2000-STY2002 (60) STY3639, STY4609, STY4543, STY2701, STY2244, STY0417 (61)	**Preliminary reports (Preclinical phase)**
	mRNA Profiling and microarray	RNA transcriptional profiling of peripheral blood to characterise host response to typhoid infection	431 host transcripts abundant in acute typhoid patients (genes encoding intracellular proteins, proteins involved in innate & adaptive immunity etc.) (98) Transporter genes ABCA7, ABCC5 & ABCD4 & ATPase were enriched (99)	
Metabolomics	Two-dimensional gas chromatography with time-of-flight mass spectrometry (GCxGC/TOFMS)	GCxGC/TOFMS on plasma from patients with S. typhi and S. paratyphi A infections and asymptomatic controls	306 metabolites separated the controls from the S. Typhi infections (2,4dihydroxybutanoic acid, phenylalanine, and pipecolic. Etc.) (57)	
		GCxGC/TOFMS on plasma from patients with culture confirmed typhoid fever, clinically suspected typhoid, and other febrile diseases (malaria)	24 metabolites to identify typhoid fever patients including glycerol-3-phosphate, stearic acid, linoleic acid, pyruvic acid, and creatinine (58)	
Genomics	Comparative Genomics and PCR	Genomic comparison of S. typhi with other enteric pathogens, in silico analysis revealed 6 specific genes; STY0307, STY0201, STY0322, STY0326, STY2020, and STY2021	5 PCR assays using STY0307, STY0201 STY0322, STY0326, STY2020, and STY2021 were developed and found to be highly specific at single-gene target resolution (59)	STY3007, STY0201, STY0207: Fluorescence polarisation based nucleic acid test (96) STY3007, STY0201; Magneto-DNA nanoparticle system (97) Preliminary reports (Preclinical phase)

[Table/Fig-2]: Next generation approaches for typhoid biomarker identification [18,58-73].

Development of new tests: A next-generation serology-based test appears to be the most simple and achievable that which could be modified to a POC format. RDT with a dipstick or immunochromatographic formats score well over conventional formats in rapidity, simplicity, requirement of minimal sample manipulation and must be considered while developing a diagnostic assay [85]. For typhoid diagnosis, TUBEX and Typhidot fare better than conventional microbiological culturing and Widal test due to their desirable operational features but are found to be unreliable in the endemic regions [9,86].

RDTs based on immunochromatographic format; TyphiDot Rapid (TR-02), Enterocheck, and OnSite Typhoid IgG/IgM Combo Rapid Test performed well upon evaluation and exhibited high diagnostic accuracy, along with operational features of rapidity, affordability, user-friendliness and easy interpretation [40,42,43]. All these tests are robust and can be used at the outpatient clinics and primary health centres requiring minimal sample volume. However, they warrant rigorous evaluation in different settings. Moreover, similar platforms can employ more specific antigens to improve their diagnostic accuracy.

Although, minimally invasive, blood can be replaced with non-invasive sample sources such as saliva, stool, and urine [30]. Salivary IgA against *S. typhi* antigens have been observed during acute typhoid (~19 mg/mL) [68,87], saliva is devoid of the Rh factor; thereby, improving the specificity. LPS and HlyE-specific salivary-IgA antibodies have been detected efficiently in acute infection but large-scale studies are required [69,88,89]. One study has utilised a recently published proteome microarray data [90] to determine the diagnostic utility of haemolysin E (HylE, t1477) antigen, as well as LPS for typhoid fever. The study shows that the HylE and LPS-

specific IgA ELISA distinguished well between typhoid patients and healthy controls [18]. Moreover, LPS specific IgA seems to be a useful indicator of acute infection due to its transient appearance during infection. The use of IgA or LPS for diagnosis is not novel, but together they function as a good marker for acute typhoid. Additionally, Typhoid-Paratyphoid Test (TPT), which involves detection of IgA antibodies against *S. typhi* in patients' blood, using ALS based assay, has shown promise. It takes advantage of infection-induced systemic migration of activated lymphocytes for the detection of antigen-specific lymphocytes in circulation [91]. The TP Test exhibited significant sensitivity (100%) and specificity (78-97%) when evaluated in Bangladesh and outperformed commonly used RDTs [19,20]. It has been used in both ELISA and immunodot format, suggesting that a lateral flow or dipstick format is possible. Although, long incubation period limits its use as a POC test, studies show that it could be performed in resource-poor regions, requiring just 1 mL of blood with minimal expertise, and lab facilities, showing its immense utility if it is developed further.

Test metrics: The test performance can be influenced by variables like population characteristics at different settings, disease prevalence and genetic variability of the host and pathogen. This also includes characteristics like the physical format, nature of antigen/antibody employed, and the skills needed to perform a test. Therefore, we need a standardised and unbiased selection process to determine the field-feasibility of a diagnostic test in a setting.

Although, there is a lack of well-defined quality standards in development and evaluations of typhoid diagnostics [92], the "ASSURED" criteria proposed by WHO [93], can be extended to typhoid diagnostics. Some key metrics that can be followed while developing superior typhoid diagnostics have been briefly described. Operationally, an ideal diagnostic test must be rapid, to allow early initiation of treatment. It must be field-deployable, cost-effective, non-invasive and require minimal infrastructure and skilled personnel. Other features such as test throughput, heat-stability of reagents, quality control, portability, simplicity of performing and evaluating the test should be assessed consistently. Technical metrics such as high diagnostic accuracy, identified by high sensitivity and specificity (100% or approaching that of the gold standard) and reproducibility are required. Furthermore, a high Negative Predictive Value (NPV) and a Positive Predictive Value (PPV) that make a test reliable and allow for accurate diagnosis are must [85].

Test evaluation: The quality of studies evaluating typhoid RDTs has been suboptimal [94]. A major barrier to the development and evaluation of new tests is the lack of an appropriate gold standard; as errors in determining sensitivity and specificity arise if the comparator is not sensitive and specific enough [95]. The highest grade of reference standard is the bone marrow culture or blood PCR in addition to the blood culture [94]. A Composite Reference Standard (CRS) could help in determining the diagnostic accuracy in the absence of a single reference standard [96]. Moreover, the establishment of bio-banks housing confirmed patient samples and its availability to researchers is essential for evaluation of new tests [97].

CONCLUSION

S. typhi is an immuno-modulatory pathogen which evades the host immune defenses and delays clinical presentation. This temporal gap between infection and disease acts as a confounder to early typhoid diagnosis. The transient presence of bacteria in the blood makes a positive blood culture challenging. Additionally, several other pathogens can cause symptoms clinically similar to typhoid fever, thus affecting timely and accurate diagnosis and appropriate treatment which may contribute to antibiotic resistance. High-throughout technologies have expanded the understanding of *S. typhi* pathogenesis. It is crucial to channel these towards diagnostic development through identification of potential biomarkers. Identified biomarkers like HlyE and CdtB have shown good diagnostic potential during preliminary evaluation. An ideal typhoid diagnostic test should be affordable, simple, rapid, non-invasive, and require minimal laboratory infrastructure, and skills. RDTs follow these metrics and could serve as ideal typhoid diagnostic tests provided desired sensitivity and specificity is achieved. Utilisation of *S. typhi*-specific IgA responses of typhoid patients in TP Test is another promising approach. Furthermore, alternate markers such as infection-specific metabolic signatures could form the foundation of new RDTs. However, clinical validation and rigorous field evaluations of the new tests and identified biomarkers needs to be done under harshest of conditions at the developmental stage for confirming the robustness of the test. This demands an accurate reference standard, thus necessitating the use of a composite reference standard and establishment of well-characterised bio-banks. Further, researchers, policymakers, and other stakeholders must come together to bridge the gap between the development of improved diagnostics, their evaluation, and implementation.

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