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REVIEW

The laboratory diagnosis of typhoid fever

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Abstract
Typhoid fever is a serious bacterial infection. Given its non-specific clinical presentation the laboratory diagnosis is critical. This article discusses existing and potential diagnostic methods. Culture particularly of blood remains very important but needs to be optimised in terms of volume and timing. Existing serological tests including the outdated Widal test and other serological tests generally lack sufficient sensitivity and specificity, though newer serological tests detecting immunoglobulin A to lipopolysaccharide, haemolysin E and other novel antigens, hold potential. Multiplex polymerase chain reaction on blood following a short pre-enrichment step also appears to offer excellent sensitivity and specificity but may be more suitable for research studies at this stage. The limitations of blood culture as the existing reference method are noted, and alternatives discussed.

Introduction

Typhoid fever is a serious bacterial infection affecting in 2010 an estimated 11.9 million people globally and causing an estimated 128 775 death.[1] The disease occurs mainly in low- and lower middle-income

countries (LMIC) in Asia and Africa and although the majority (> 70%) of cases occur in Asia, the incidence of disease may be highest in Africa with estimated rates of up to 537-557/100 000 population in East and Middle Africa.[1]

Typhoid fever is an epidemic-prone disease. Review of global data from 1990 -2018 shows that the number and size of typhoid fever outbreaks are increasing over time particularly in LMIC. The average size of outbreaks is much larger in Africa compared to other regions, suggesting late detection.[2] There have been several outbreaks in Africa since 2010, predominantly in southern and central Africa, largely due to a specific multi-drug resistant (MDR) strain or haplotype called H58 which appears to have emerged from Asia and spread globally.[3]

Typhoid and paratyphoid fever are caused by infection with *Salmonella enterica* serotype *Typhi* and *Salmonella enterica* serotypes *Paratyphi A, B* or *C* respectively.[4] These 'typhoidal' and 'paratyphoidal' serotypes infect only humans. In contrast the 'non-typhoidal' Salmonella serotypes tend to be associated with a variety of animal hosts and in humans typically cause gastro-enteritis though invasive disease can occur in the immunocompromised.[4] Paratyphoid fever is similar clinically to typhoid fever. It is thought to be less severe, though this has been challenged.[4,5] At present it is much less common than typhoid fever and seems to be rare outside of Asia where *S. paratyphi A* accounts for up to 20% of cases. The term enteric fever encompasses the clinical syndromes of both typhoid and paratyphoid fever though the term typhoid fever is sometimes used interchangeably, with the understanding that *S. paratyphi* serotypes may actually be the causative organisms.

The clinical features of enteric fever are non-specific.[6] In essence enteric fever presents as a prolonged undifferentiated febrile illness. Fever is a consistent finding in patients of all ages, though chills and rigors are much less common in children than in adults.[6] The classical early clinical signs of typhoid fever such as relative bradycardia and rose spots may be absent.[6] Other common non-specific early symptoms include headache, nausea, vomiting, constipation, diarrhoea, cough and abdominal pain.[6] Hepatomegaly and splenomegaly also occur frequently.[6]

Gastro-intestinal complications such as bleeding, granulomas/abdominal masses or perforation may occur in the 2nd or 3rd week, more frequently in older children or adults compared to young children, since these reflect a robust immune response to well primed Peyer's patches.[7] Transient pancytopenia is a classical feature of typhoid fever. While this can occur in children, leucocytosis also occurs, particularly among young children.[7] Other features more commonly found in young children < 5 years of age include anaemia, hepatitis and seizures.[6,7] Apart from variation with age, there may also be geographic variation in nature of some presenting signs and symptoms, e.g. relative bradycardia is reported in 50% of cases in sub-Saharan Africa compared to less than 20% of cases elsewhere.[6]

The differential diagnosis of typhoid fever can be extensive including bacterial sepsis, malaria, acute abdomen and other locally relevant febrile illnesses, such as dengue, brucellosis or rickettsial infections.[6]

The laboratory diagnosis is therefore critical to the accurate diagnosis and management of the individual patient as well as for an improved understanding of the epidemiology of the disease, and for limiting unnecessary antibiotic use as part of antimicrobial stewardship. This article will discuss the different options for laboratory diagnosis and suggest future directions and key actions.

Culture

Culture of *S. typhi* or *S. paratyphi* from sterile sites provides a definitive diagnosis of typhoid fever.[4] In addition, culture permits antimicrobial susceptibility testing and molecular typing that guides treatment and informs public health and infection control activities.

Blood is the preferred specimen type though stool or urine samples may yield positive results in about 30% of patients in the second week of illness.[4] Theoretically bone marrow culture is still considered the gold standard as the sensitivity is higher, up to 90% and less affected by prior antimicrobial therapy,

but bone marrow is not a practical sample to collect in virtually any clinical setting. The sensitivity of blood culture for the diagnosis of typhoid fever is reported to be about 60%.[8] This modest sensitivity may be related to the low bacterial density encountered in typhoid fever where median *S. typhi* counts in blood may be only 1 ml (CFU/ml).[9]

Other factors apart from prior antimicrobial therapy that influence blood culture yield include duration of illness, as sensitivity is greatest in first week of illness, as well as the volume of blood sampled. Many studies either fail to document the volumes of blood cultured or use insufficient volumes. A recent systematic review and meta-analysis suggested that sensitivity increases by 3% for every 1 ml increase in blood volume between 1ml – 10ml.[8] A minimum volume of 7ml has been suggested as it should give a 90% chance of detection if median bacterial load is 0.3 CFU/ml, although ≥ 10ml may be necessary.[10]

The optimal volume for blood culture in general in children has been labelled a conundrum.[11] A number of age or weight-based recommendations exist but these differ considerably from each other e.g. for a child of 8 kg recommended volumes vary between 1.5ml and 10 ml.[11] In general, given the low bacterial density of typhoid fever, using volumes at the higher end of the recommended ranges would be preferred. Outside of the neonatal period (an age at which typhoid fever is rarely a concern) these volumes are all less than 1% of total blood volume, and so should be safely tolerated in most children.[11] In practice smaller volumes are frequently used, e.g. a recent community based prospective paediatric cohort study met their protocol specified requirements to collect at least 3ml in children ≤ 3 years and at least 5 ml in older children from 3 - 15 years of age and yet showed that a larger blood volume inoculated for culture was independently associated with blood culture positivity with an Odds Ratio (OR) of 2.82 (95% CI, 1.71–4.66).[12]

The particular laboratory methods used for culture of different specimen types may also play a role in successfully isolating the organism, e.g. for blood culture the medium should be a rich nutrient broth containing a lysing agent while the absence of automated blood culture systems in LMIC laboratories may contribute to a low yield and prolonged time to positivity.[13] The yield from stool culture may be increased by testing of multiple samples and the use of an enrichment step such as selenite broth.[4]

The isolation and identification of causative organisms is complex and requires appropriate laboratory protocols such as the use of selective media for non-sterile sites and multi-step algorithms for identification that include biochemical methods and serotyping. Culture has a slower turnaround time compared to serology or molecular methods, while newer tools introduced into diagnostic laboratories to speed up identification such as mass spectroscopy (MALDI-TOF) do not differentiate enteric fever pathogens from other *Salmonella* species. Biosafety protocols are particularly important to prevent laboratory acquired infections among staff working with concentrated cultures of live organisms.[14]

Molecular methods

Theoretically molecular methods such as polymerase chain reaction (PCR) should be extremely sensitive and specific, and a number of PCRs have been developed. Initially these targeted *S. typhi* genes encoding known antigens such as flagellar H[15] and Vi antigens[16] but subsequently have expanded to encompass a wider range of unique targets in *S. typhi* and *S. paratyphi.*[17,18] In practice the sensitivity, typically in comparison to the imperfect reference methods of culture or a mixture of culture and serology, has been disappointing at a maximum of approximately 90%. This is possibly due to the low bacterial load in blood where the median *S. typhi* count is 1 CFU/ml or lessc[9,10] compared to the lowest limit of detection of many PCR assays of 4 CFU/ml.[10] The need for a multiplex PCR that also simultaneously identifies and differentiates *Salmonella paratyphi A* and invasive non-typhoidal *Salmonella* species aggravates this problem as multiplex PCRs tend to perform worse than conventional single target ones.[17]

Methods to increase sensitivity for the detection of *S. typhi* have been described, such as nested PCR,[20] as well as methods to enrich bacterial DNA concentrations by removing human DNA[21] and or by including a short period of incubation to permit bacterial growth before DNA extraction.[22] Recent use

of such a pre-amplification incubation step together with a multiplex PCR for the detection of *S. typhi* and *S. paratyphi* A in patients presenting with a febrile illness of at least 3 days without a clear focus of infection showed an overall sensitivity and specificity > 92% when compared to optimised blood culture using verified though generally low blood volumes in an automated blood culture system.[19] A similar approach in Malawi in young children presenting with undifferentiated fever to a tertiary hospital showed an increase in case detection of 62-94%.[23] Molecular methods can also be applied to gut tissue samples collected at surgery in cases with intestinal complications where prior antibiotics and presence of normal gut flora may hinder culture based detection.[24]

Additional drawbacks of molecular tests include complexity, cost, and lack of susceptibility results. Turnaround time may be reduced compared to culture, provided testing is done on demand rather than being processed in batches or referred to a distant central laboratory. A simpler more affordable molecular method involving pre-concentration followed by Loop-mediated isothermal amplification (LAMP) has been described[25] and similar developments may overcome some of these drawbacks in the future.

Serology

In settings where microbiological investigations and culture are not easily available the diagnosis of typhoid fever is often based on serology, notably the Widal test, first described in 1896.[26] The Widal test is an agglutination test that detects antibodies in sera to O (Lipopolysaccharide (LPS)) and H (flagellin) antigens of *S. typhi*.[27] It is a rapid, inexpensive, and simple test. Theoretically the test should be repeated after 7- 10 days to demonstrate a four-fold rise in titre of antibodies, but usually interpretation is based on a single test performed at the time of presentation.[28] There is considerable variability in the performance of the test and this lack of standardisation results in poor reproducibility.[26]

The Widal test lacks both sensitivity and specificity. False negatives may occur early in disease, false positives may reflect previous infection or vaccination, or cross reactivity with antibodies resulting from other infections, including non-typhoidal *Salmonella* or other bacteria such as *Brucella*.[27] In endemic areas varying cut-offs have been used to determine positivity, ideally based on contemporary baseline values in the local community. Cut-off titres in use typically range from 1: 40 – 1:200.[29] However, even with customised cut-offs, Widal test results should be interpreted with caution, e.g. in a study of hospitalised adults in Vietnam with a very high prevalence of typhoid fever of approximately 30% in a setting where non-typhoidal salmonella infections and vaccination were rare and using moderately high cut-off titres of > 1: 200 or > 1: 100 for O and H antigens respectively, the positive predictive value (PPV) and negative predictive value (NPV) were only 86% and 90% respectively.[30] In general the use of the Widal test for the diagnosis of typhoid fever is not recommended.

Several alternative serological tests have been developed over the years. A Cochrane review in 2016 of commercially available rapid diagnostic tests included 37 studies with more than 5000 participants mainly from highly endemic areas in Asia. Sixteen different RDTs were assessed although 3 assays and their variants (TUBEX, Typhidot and Test-it Typhoid) constituted the majority of studies. The review reported only moderate diagnostic accuracy with sensitivities ranging from 70- 85% and specificities from 80-90% with no evidence of a difference between the average performance of the 3 common tests.[31]

More striking was the overall heterogeneity of results, and the overall poor quality of studies, e.g. most studies selected patients on the basis of a clinical suspicion of typhoid fever although the criteria for such suspicion were usually not stated, and less than one third of studies recruited patients presenting simply with fever, the patient population in whom the diagnostic test would most likely be applied. In addition,16 of 37 studies used a case control study design which is likely to overestimate diagnostic accuracy. Similarly, only 3 of 37 studies used bone marrow culture as part of the reference method. The systematic review concluded that none of the tests included were sufficiently accurate to replace blood culture, and that more robust evaluations of alternative RDTs were required. Other systematic reviews have highlighted similar concerns about the quality and heterogeneity of studies of alternative

diagnostic tests, noting that key details regarding the reference test used, such as the volume of the biological sample cultured and the receipt of prior antibiotics, are not recorded in most studies.[32,33]

More recently various new techniques have been used to identify novel targets for use in next generation serodiagnostic tests.[34-37] The targets identified include LPS, haemolysin E (HlyE), cytolethal distending toxin B (CdtB), membrane preparation (MP), flagellin and various others. Tests detecting IgA isotype antibodies also seem to be more accurate compared to those detecting IgG or IgM antibodies, possibly because IgA is more transiently produced compared to long lasting IgG but is more specific and less prone to cross reactions than IgM.[35,38]

Recently a rapid point of care (POC) immunochromatographic test was developed using an existing commercial Dual Path Platform (DPP) technology that includes separate paths for sample and for conjugate, together with a portable digital reader.[39] Based on detection of IgA to HlyE and to *S. typhi* LPS, an initial evaluation of its use in a small group of adults in Bangladesh reported a sensitivity of 90% and specificity of 96% (compared to febrile endemic controls). Further studies are required, but this POC test could be a major improvement because of the rapid turnaround time, ease of performance and no requirement for specialised equipment or highly trained laboratory staff.

Future directions

To guide researchers and developers, detailed specifications for an improved typhoid diagnostic test have already been suggested.[40] Based on a literature review, a Target Product Profile (TPP) including both the minimal and optimal characteristics for 36 parameters, was drawn up and subsequently refined using 2 rounds of a Delphi survey with key stakeholders and experts. Among the main requirements for the TPP were that the target population should include patients of all ages presenting with undifferentiated febrile illness at any point in the disease to the lowest level of the health care system. The test should cover both *S. typhi* and *S. paratyphi*, require <1 ml of blood, cost ideally only $1 and have a sensitivity of around 90% and specificity ≥ 95%. Ideally such a POC test also needs to be part of a diagnostic and treatment algorithm that caters for other locally relevant febrile infectious diseases.

Part of the difficulty in developing and evaluating alternative diagnostic tests for enteric fever is the imperfect reference standard provided by culture of blood, or even of bone marrow. Using an imperfect reference standard might reduce the specificity of a novel diagnostic test i.e. the so-called "false positive" might actually be a true positive.

Potential solutions to this problem include the use of a composite reference standard or the use of a Bayesian approach with latent class analysis. A composite reference standard (CRS) combines multiple tests with good specificity thereby increasing the overall sensitivity, e.g. positive culture from blood or bone marrow or urine, or positive culture and/or positive PCR. The advantages of a CRS compared to other alternative reference standards are that it can be more clearly defined and is more straightforward to interpret. A recent meta-analysis motivated for the development of a CRS for Typhoid fever using clear standardised definitions to facilitate good quality prospective cohort studies of new diagnostics.[32] Another meta-analysis using a Bayesian network procedure with latent class analysis grouped tests according to the underlying principle i.e. antibody detection, antigen detection, PCR, etc., and despite the similar problem of highly heterogeneous studies was able to draw some broad conclusions as well as establishing a method for evaluating the performance of combinations of tests.[33]

Other novel diagnostic approaches are also being explored for the diagnosis of typhoid fever including proteomics,[41] transcriptomics[42] and metabolomics[43] though none have yet been translated into tests for routine clinical use nor are they likely to meet the Target Product Profile requirements in the near future.

Conclusions

There is a need to improve the laboratory diagnosis of typhoid fever. To maximise benefit from existing test methods, it is important to:

1. Optimise the yield from blood cultures by ensuring the collection of adequate volumes of blood- at least 7ml in adults, and at least 3ml in children ≤ 3 years and at least 5 ml in older children from 3 - 15 years of age. Cultures should also be taken at the time of presentation before the administration of antibiotics.

2. Ensure that routine diagnostic microbiology laboratories are capable of the complex tasks required for the culture-based isolation, identification and susceptibility testing of typhoidal *Salmonella* species from blood and other specimens. This might mean ongoing support in terms of adequate numbers of appropriately trained staff, development of updated standard operating procedures and test algorithms and a reliable supply of the necessary media and reagents. Smaller laboratories need to be supported by larger reference or public health laboratories who also play an important part in the control of outbreaks of typhoid fever by providing timeous and accurate surveillance reports, including ideally molecular typing of strains.

3. In areas where the Widal test remains the only practical diagnostic test available currently, its performance and interpretation should be critically assessed, and results applied with caution. Health systems need to ensure that patients at different levels of care have access to diagnostic tests for typhoid fever.
   Improved diagnostic tests are also urgently required.

4. In particular, there is a need for a POC test with improved performance characteristics for use particularly among outpatients. Ideally this POC test should meet the suggested requirements of the Target Product Profile. It is not clear yet whether the Dual Path Platform POC test targeting IgA to HlyE and to LPS meets these requirements. However, the identification of a variety of other novel antigens and host immune responses holds potential for development of alternative rapid tests.

5. Molecular tests that offer improved sensitivity whilst also targeting and differentiating *S. typhi* and *S. paratyphi* as well as non-typhoidal salmonella species are required. While such tests may not be practical as routine diagnostic tests, they may be useful in research settings like epidemiological studies in high disease burden settings and as secondary endpoints in vaccine efficacy studies. Simpler molecular methods using LAMP amplification hold potential for future use as tests that can be performed closer to patients in smaller laboratories.

6. Studies of future potential diagnostic tests should be of high quality including improved study design, optimisation of blood culture methodology and accommodation of an imperfect reference standard.

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