Specimens and culture media for the laboratory diagnosis of typhoid fever

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Abstract

Background: Culture of S. Typhi is necessary for the definitive diagnosis of typhoid fever and provides isolates for antibiotic susceptibility testing and epidemiological studies. However, current methods are not fully optimised and sourcing culture media and bottles for culture media may be problematic.

Methodology: In two hospital laboratories in Viet Nam, comparisons of media for blood and stool culture were conducted. The effect of the volume of blood or stool on culture positivity rate was examined and direct plating of the blood buffy coat was trialed.

Results: For 148 suspected typhoid fever cases, ox bile broth (58 positive) and brain-heart infusion broth containing saponin (63 positive), performed equally well. For 69 confirmed adult typhoid fever cases, large-volume (15ml) blood culture gave the same sensitivity as 1 ml of bone marrow culture. For 44 confirmed typhoid fever cases, the direct plating of the buffy coat was positive in 28 cases. For 263 positive stool cultures, selenite F and selenite mannitol performed equally well and culturing 2 g rather than 1 g increased the isolation rate by 10.5%.

Conclusions: For the diagnosis of typhoid fever by blood culture the medium should be a rich nutrient broth containing a lysing agent. In adults 1 ml bone marrow or 15 ml blood culture gave similar results. Where isolates are needed for susceptibility testing or epidemiological studies, but resources for culture are scarce, direct plating of the blood buffy coat can be used with a 50% fall in sensitivity compared to standard blood culture.

Key Words: S. Typhi, typhoid fever, epidemiology


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Introduction

Typhoid fever is a prolonged and debilitating fever which is of major public health concern in many countries [1]. There are many other causes of prolonged fever in typhoid endemic regions [2]; therefore, the clinical diagnosis of typhoid fever is problematic [3] and laboratory tests are necessary. The isolation of S. Typhi from blood or bone marrow is diagnostic of typhoid fever [4, 5] and stool culture is important for monitoring the carriage of S. Typhi and may also help diagnosis [6]. Serology is available but has low sensitivity or specificity [7]. Culture of the causative organism remains the most effective diagnostic procedure in suspected typhoid fever and the optimisation of culture methodology is therefore important. For blood culture, published reports suggest that a 10% suspension of ox bile is the best medium [8]. It has the advantage of inhibiting the growth of skin contaminants, but it also inhibits the growth of most other blood-stream pathogens and therefore cannot be used as a routine diagnostic test for bacteraemia. The isolation of S. Typhi after bone marrow aspiration, a highly invasive procedure, is considered to be the gold standard method for the diagnosis of typhoid fever and is reported as more sensitive than blood culture by most, [9,10] but not all, authors [11]. We have previously quantified the number of bacteria in blood and bone marrow and found that the enhanced isolation of S. Typhi from bone marrow can be explained by the larger number of bacteria found, which is tenfold more per volume of bone marrow than per volume of blood [12]. However, if enough blood is cultured it may be possible to increase the sensitivity of blood culture to that of bone marrow culture, thus avoiding the need for bone marrow aspiration.
For stool culture, enrichment media containing selenite have been shown to be more effective than other available enrichment media [13,14]. Selenite with mannitol (selenite M) has been recommended [15] but there have been no large-scale comparisons of different selenite containing enrichment broths. As selenite F media is the gold standard for the isolation of Salmonella from stool, a comparison of selenite F and selenite plus mannitol is necessary before the latter should be considered for use.

In many diagnostic laboratories, blood culture media, or often the bottles for the blood culture media, may not be available. The direct examination of buffy coats has been used for the detection of bacteria from blood [16] but has been reported as being of little value [17] because of operator dependent errors. The culture of lysed [18] and centrifuged blood [19], however, has been very successful. The direct culture of buffy coat, previously shown to contain almost all S. Typhi found in blood [20], should therefore allow the isolation of S. Typhi without the need for blood culture media or bottles.

The diagnostic utility of different media and specimens was studied in the following comparisons: selenite F with selenite versus mannitol as media for stool culture; ox bile broth (Oxgall) versus brain heart infusion broth as media for blood culture; the culture of 15 ml blood culture versus 1ml bone marrow; the culture of 1gm or 2 gm of stool direct plating of blooduffy coat versus a 5-ml blood culture.

Materials and Methods

Patients

All patients were admitted to the Centre for Tropical Diseases (CTD), Ho Chi Minh City, or the Provincial Hospital of Dong Thap, Vietnam, between 1993 and 1999. All patients had a clinical diagnosis of enteric fever on admission. All work on blood and bone marrow cultures was conducted at the Dong Thap hospital. Stool culture work was performed at both sites.

Identification of S. Typhi

S. Typhi from all specimens was identified by the same standard methods in the two hospital laboratories. Agglutination was performed upon primary isolation with antisera against O9 and Vi antigens (Murex, Dartford, UK). Biochemical testing was then performed using Kligler iron agar, urea slopes, citrate utilisation media, SIM motility media and MR media (all from Oxoid, Basingstoke, UK).

Investigation of blood culture media.

Quantitative blood cultures were conducted using 9 ml of blood by a pour plate method. Three 1-ml (0.5 ml in small children) aliquots of blood were mixed with 19 ml of molten Columbia agar (Oxoid, Basingstoke, UK) at 50°C; three were mixed with Columbia agar containing 0.05% sulphopolyanethosulphonate (SPS); and three were mixed with an equal volume of 0.1% digitonin (Sigma, Poole, UK) and incubated at 37°C for 15 minutes before mixing with Columbia agar plus SPS. After being allowed to set, all nine plates were incubated at 37°C for four days. Bacterial counts were recorded as colony forming units (CFU) per milliliter of blood. Up to 5 colonies were picked from the surface of the agar to confirm the identification as S. Typhi.

Three blood culture media were compared: BHISPS [brain-heart infusion broth (Oxoid, Basingstoke, UK) with 0.05% SPS (Sigma)], BHISAP [brain-heart infusion broth with 0.05% SPS plus 0.05% saponin (Sigma)], and ox bile broth (Oxgall, Difco, Detroit, USA). Blood was collected (15 ml from adults, 7.5 ml from small children) for culture before treatment was started and was divided equally into 3 bottles containing one of the above media. Bottles were subcultured daily and plated onto sheep blood agar base (Oxoid, Basingstoke, UK) containing 5% sterile sheep blood obtained from the Centre for Tropical Diseases, Ho Chi Minh City, Vietnam. For comparison of blood culture with bone marrow culture, at least 1ml of bone marrow was aspirated from the iliac crest on the following day.

Investigation of stool culture

A comparison of selenite F and the recommended selenite with mannitol (selenite M) was performed. Up to three stools were taken on admission or on consecutive days following admission, and approximately 1 gram of each stool was inoculated into 10 ml of either selenite F or selenite M (Oxoid, Basingstoke, UK). Stool specimens were inoculated into one or two bottles of each media. All media were prepared following the manufacturer’s instructions. Each batch was quality controlled for pH and differential enrichment of S. Typhi in the presence of Escherichia coli. Broths were incubated for 24 hours at 37°C. The depth of the broth was 2.5 cm. A standard bacteriological loop was used to culture 5µl from the top of the broths onto MacConkey and XLD agars (Oxoid, Basingstoke, UK) after 18 –24 hours incubation. Isolates which had been previously grown from only one of the two selenite broths were tested for
their ability to grow in both broths by inoculating 10⁴ S. Typhi together with exactly 0.5 g of faeces from a healthy volunteer into 10 ml of each of the two broths. The stool from the healthy volunteer was tested for anti-S. Typhi activity by placing 1 g of faeces on a lawn of S. Typhi and 2 gm were inoculated into each of 20 ml of selenite F and selenite M to check for growth of S. Typhi. The selenite broths were subcultured at 18 and 24 hours.

Quantitative stool culture was carried out as follows: one gram of stool was emulsified in 10 ml of selenite M and left to stand for 30 seconds and 1μL and 10μL were then plated onto XLD agar using plastic disposable loops and spread for counting.

Reduced volume blood cultures and direct plating

To investigate the rapid culture of S. Typhi from blood,uffy coat from up to 5 ml of heparinised blood was collected from patients and transported immediately to the laboratory. The blood was centrifuged at 4,000 rpm for 5 minutes and the buffy coat collected using a sterile 1 ml syringe. The volume of buffy coat was 0.1ml in each case. Buffy coats were then selected alternately to be inoculated into 10 ml of Oxgall or spread onto the surface of a Columbia agar plate containing 0.05% saponin. Broths were incubated at 37°C and sampled after overnight incubation and again after 2 days onto sheep blood agar base (Oxoid, Basingstoke, UK) containing 5% sterile sheep blood (CTD). Plates were incubated and read after overnight incubation at 37°C.

Statistical methods

To test the null hypothesis that positive or negative culture results from one media were within the limits of variation expected by chance, McNemar’s χ² test for paired proportions was used to analyse discordant pairs. More than a 5% difference in positives from the media under comparison was considered to be clinically important.

Results

Investigation of blood culture media

To investigate the quantitative effects of the inhibition of antibacterial activity by SPS and of lysis of blood cells on the isolation of S. Typhi from blood, a set of 3 quantitative blood cultures (QBC) were performed on blood from 110 patients of which 91 patients were culture positive for S. Typhi. The mean (SD) counts were as follows: Columbia agar, 17.4 (52) CFU/ml; Columbia agar plus SPS, 19.9 (57.5) CFU/ml; and Columbia agar plus SPS plus digitonin, 51.2 (220) CFU/ml. The variation in counts was very high and so the differences did not reach statistical significance.

A set of 3 blood cultures, BHISPS, BHISAP and Oxgall, were processed from 148 patients with suspected typhoid fever. Oxgall and BHISAP had a higher positive culture rate on Day 1 than did BHISPS, but the difference in overall isolation rate between these broths was not significant (Table 1).

<table>
<thead>
<tr>
<th>Media</th>
<th>BHISPS Positive cases (%)</th>
<th>BHISAP Positive cases (%)</th>
<th>Oxgall Positive cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive by Day 1</td>
<td>38 (25.7)</td>
<td>48 (32.4)</td>
<td>52 (35.1)</td>
</tr>
<tr>
<td>Positive by Day 5</td>
<td>60 (40.6)</td>
<td>63 (42.6)</td>
<td>58 (39.2)</td>
</tr>
<tr>
<td>Positive by Day 10</td>
<td>60 (40.6)</td>
<td>63 (42.6)</td>
<td>58 (39.2)</td>
</tr>
<tr>
<td>Negative at Day 10</td>
<td>88 (59.4)</td>
<td>85 (57.4)</td>
<td>90 (60.8)</td>
</tr>
</tbody>
</table>

BHIS, Brain heart infusion broth. SPS, sulphopolyanetholesulfonate (0.05%) SAP, saponin (0.05%) Oxgall is 10% ox bile.

There were 85 patients with culture positive typhoid fever by any method. The isolation rates of S. Typhi from BHISAP and Oxgall were directly compared in these patients (Table 2). There was no significant difference between these broths. The contamination rates for each broth were recorded and, as expected, ox bile broth had the least contamination; BHISPS: 2.2%, BHISAP 4.5%, Oxgall <1%. Contaminating organisms were always coagulase negative staphylococci or aerobic spore-bearing bacilli.

<table>
<thead>
<tr>
<th>BHISAP positive</th>
<th>BHISAP negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxgall positive</td>
<td>58</td>
</tr>
<tr>
<td>Oxgall negative</td>
<td>9</td>
</tr>
</tbody>
</table>

* χ² = 0.64 (p= >0.2). * positive by 15ml blood culture or bone marrow culture. BHISAP is brain heart infusion broth with sulphopolyanetholesulfonate (0.05%) and saponin (0.05%)

Reduced volume blood culture

The use of the buffy coat to isolate S. Typhi was investigated as an alternative to culture of whole blood. Positive results were obtained in 20/28 (71.4%) buffy coats collected from 28 blood culture positive patients and cultured in 10 ml of Oxgall broth. A direct comparison between a 5-ml whole blood culture in
BHSPS and a Buffy coat culture in 10 ml Oxgall was then performed with samples from 67 patients. The results are presented in Table 3. Buffy coat culture was less sensitive than blood culture as expected, but did produce colonies in 70% of cases that had a positive blood culture.

Table 3. Comparison of broth culture of Buffy coat from 5 ml of whole blood with a standard 5-ml blood culture. All patients had culture-proven typhoid fever.

<table>
<thead>
<tr>
<th>All cultures from 5 ml of blood</th>
<th>Buffy coat culture positive</th>
<th>Buffy coat culture negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood culture positive</td>
<td>24</td>
<td>15</td>
</tr>
<tr>
<td>Blood culture negative</td>
<td>4</td>
<td>3*</td>
</tr>
</tbody>
</table>

*Positive by 15 ml blood culture but negative by 5 ml blood culture.

Comparison of blood culture and bone marrow culture

A 1-ml bone marrow culture gave a significantly higher isolation rate for S. Typhi than did a 5-ml blood culture (Table 4a); however, no difference could be detected between a 15-ml blood culture and a 1-ml bone marrow culture (Table 4b). These observations suggest that increasing the volume of blood cultured to 15 ml improves the sensitivity of blood culture to that of bone marrow.

Table 4. Comparison of 5 ml and 15 ml blood culture with 1 ml bone marrow culture for culture-proven typhoid patients.

<table>
<thead>
<tr>
<th>Bone marrow culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood culture, 5 mL</td>
</tr>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>Blood culture, 15 mL</td>
</tr>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
</tr>
</tbody>
</table>

*χ² = 6.1; p < 0.02. #χ² = 0.06; p > 0.2

Investigation of stool culture

S. Typhi was grown from 263 stools that had been inoculated into selenite F and selenite M broths. There were 208 positive results for both broths. Of these, 27 were positive only in selenite F and 28 were positive only in selenite M. There was no detectable difference in the isolation rate of S. Typhi from stool between selenite F and selenite M. The 29 isolates of S. Typhi that were grown only in one type of selenite broth were investigated for their ability to grow in both broths. Seventeen of these isolates had previously only grown in selenite F and 12 had only grown in selenite M. All S. Typhi grew in both broths when re-inoculated. This observation suggests that the inability of some S. Typhi isolates to grow in only one broth was not a property of the broth. To investigate the effect of bacterial numbers on the isolation rate of S. Typhi, quantitative cultures were performed on 45 culture positive stools. For cases where S. Typhi grew from both broth cultures, the mean (standard deviation) quantitative count from 33 stools was 17,000 CFU/ml (8600); however, the quantitative plates from 12 stools that gave positive cultures in one broth only were always negative indicating less than 1 CFU/ml. The reason for growth of S. Typhi in one out of the two broths during primary isolation in this study is therefore the probability of sampling from low numbers of bacteria in the specimen.

Comparison of large volume stool culture with multiple sampling

Of 263 positive stools, where multiple aliquots were cultured from a single stool, 55 (21%) stools grew S. Typhi from a single sample. The isolation rate of S. Typhi from stool was therefore improved by 10.5 % when higher volumes of stool were cultured.

Direct plating of blood

In an attempt to remove the need for expensive broth, a 5-ml blood sample collected from each of 45 patients was spread directly onto saponin Columbia agar plates. This procedure lysed the cellular fraction of the blood containing the S. Typhi. In 23 confirmed typhoid fever cases, 10 (43%) were positive following the direct plating of the Buffy coat on saponin Columbia agar plates. The reason for negative cultures in four cases was overgrowth with Bacillus species plate contaminants. In general, contamination of the direct plate cultures was with a single colony that was easily distinguishable from S. Typhi and occurred in about 25% of cases.

Discussion

Blood culture has been the mainstay of the culture-based diagnosis of typhoid fever since 1900. In 1907 Coleman published the first review of blood cultures in typhoid fever [21] and recommended the use of ox bile broth. In 1911 its superior qualities were attributed to the inhibition of the antibacterial activity of fresh blood caused by lysis of blood cells rather than direct enhancement of growth by the bile salts [22]. In our study, lysing the blood cells with digitonin (a lysing agent known not to effect the growth of bacteria) increased the number of bacteria recovered in the presence of SPS (51CFU/ml after lysis compared with
19 CFU/ml before lysis). Thus cell lysis can increase the numbers of bacteria recovered from a given volume of blood. The reason for this may be either the release of intracellular bacteria from phagocytes allowing the bacteria from a single cell to form several colonies, or the release of hemoglobin from red cells to act as a growth factor. Although Oxgall was superior to BHI in this study, when compared with BHI plus the lysing agent saponin there was no difference (Table 2). Such a broth has been previously evaluated for use as blood culture media and has the advantage, over Oxgall, of being able to support the growth of a wide range of other bacteria and fungi [23]. Indeed, a medium capable of supporting the growth of S. Typhi as efficiently as ox bile broth but also capable of supporting the growth of a wide range of blood borne pathogens has been called for [5].

Stool culture can be a useful aid to the diagnosis of acute typhoid and we have shown that the most commonly used selenite broth, selenite F, is as good as the recommended selenite M [15], thus removing the need for laboratories to carry both broths. A rarely investigated factor in stool culture is the volume of stool cultured. It is well reported that multiple samples of stool are needed for the isolation of S. Typhi. Our results confirm this: when three stools were cultured, 31.6% of patients were positive compared with only 13.4% from a single stool. In this study we also performed multiple cultures on single samples. By culturing 2 g of stool rather than the accepted 1 g we increased the isolation rate by 10%.

As a way to reduce the need for expensive blood culture bottles and bottles we examined the use of direct plating of blood. Direct plating onto agar was positive in 43% of patients who also had a positive 15-ml whole blood broth culture.

We believe that this technique is useful for epidemiological studies in locations remote from expensive laboratory facilities. Although centrifugation is necessary for the collection of buffy coats, this can be achieved using a hand centrifuge. Storage of isolates on agar slopes at room temperature would provide strains for epidemiological studies and for susceptibility testing against relevant antimicrobial agents at reference facilities. Plasma from the buffy coat specimens could be used for biochemical and serological tests and for seroprevalence studies. Staphylococcus aureus, Streptococcus pneumoniae [16] and Neisseria meningitidis have been grown from direct cultures of blood using lysis centrifugation methods and from buffy coats. Direct culture of buffy coat, therefore, may also aid in the diagnosis of other clinical conditions such as meningitis.

**Conclusions**

We conclude that modern blood culture media, such as brain heart infusion broth with SPS, are suitable for culturing S. Typhi from blood. If a lysing agent such as saponin is added, the broth performs as well as ox bile broth. Despite the relatively small sample sizes and the fact that the effect of previous antibiotic therapy was not assessed in this study, we contend that a high-volume blood culture (15 ml) should increase the sensitivity of isolation to that of bone marrow culture. For the isolation of S. Typhi from stool, selenite with mannitol offers no advantage over Selenite F. Selenite F can therefore be used for culture of stool for all faecal salmonellae. The volume of stool cultured from each sample should be at least 2 g but higher culture volumes may increase yields.

If blood culture bottles or media are not available, direct plating of the buffy coat from 5-10 ml of blood will allow the growth of isolated colonies within 24 hours of specimen collection for around 50% of blood culture positive patients.

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**References**


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