Modification of the TUBEX typhoid test to detect antibodies directly from haemolytic serum and whole blood

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The TUBEX test for typhoid fever detects serum antibodies in a simple and rapid assay system based on the inhibition of binding between two types of reagent particles — magnetic particles coated with an antigen (Salmonella O9 LPS) and coloured indicator particles coated with an anti-O9 mAb. A magnet is used to separate the colour indicator particles bound to the magnetic particles from the unbound indicator particles. Specific colour changes following magnetic separation are indicative of antibodies in the patient’s serum; however, because results are interpreted based on changes in the colour red, haemolytic or icteric specimens cannot be used. This study describes a simple modification of the protocol to accommodate such specimens, including whole blood. This involves the addition of a quick and simple washing step after mixing the specimen with the antigen-bound magnetic particles. This modification has the advantage of allowing larger sample volumes to be used, thus enhancing the assay sensitivity, and also enables cases considered to be borderline positive by the original method to be re-assessed.

INTRODUCTION

The recently introduced TUBEX test for typhoid fever (IDL Biotech) is rapid (~5 min), simple (no washing step) and accurate (House et al., 2001; Olsen et al., 2004; Kawano et al., 2007; Rahman et al., 2007). It employs a unique assay system to detect serum antibodies to the O9 antigen found in the LPS of the causative organism, Salmonella enterica serotype Typhi (S. Typhi). IgM antibodies are detected in a reaction (Tam & Lim, 2003) enhanced by IgG antibodies (Rahman et al., 2007), and these are detectable in the first week of illness (Rahman et al., 2007). The antibodies are detected by their ability to block binding between a reagent anti-O9 mAb coupled to blue-coloured indicator microspheres and a reagent O9 antigen prepared in the form of S. Typhi LPS-coupled magnetic microspheres (Lim et al., 1998). When the aqueous suspensions of both reagents are mixed together in the absence of inhibiting antibodies, the reagent antibody particles bind to the antigen particles and together they sediment to the bottom of the reaction well when the well is placed on a magnet stand. The resulting appearance of the reaction mixture is thus colourless in the absence of any suspended particles, but, for easy visualization, the background is artificially coloured red by the presence of inert red microspheres. If, on the other hand, inhibiting antibodies are present, these will prevent binding between the two types of reagent particle to an extent dependent on the concentration of the inhibiting antibodies. A blue colour results from the unsedimented blue indicator particles, ranging from light reddish blue (low antibody concentration) to dark blue at the extremes of inhibition. The results, which are thus semi-quantitative, are read against a colour chart provided, with scores ranging from 0 (red, most negative) to 10 (dark blue, most positive).

The colour reading system of TUBEX allows easy visualization of the results, which is ideal for point-of-care use. However, there are problems when it comes to specimens that are heavily haemolytic or icteric. In addition, it is sometimes difficult to interpret results that are borderline positive. Here, we describe a modification of the existing procedure to address these problems, involving the mere addition of a simple washing step to the procedure.

METHODS

Mouse mAb. The mouse IgM mAb 3h1 used in this study for spiking was specific for the Salmonella O9 antigen (a-tyvelose) and was the same antibody as used in the TUBEX kit (not available commercially). The antibody preparation used in the study was obtained and purified from mouse ascites by cryoprecipitation in our laboratory, and the protein content was quantified using a BCA protein assay kit (Pierce).

Human serum and blood. The serum preparation used for spiking with mouse mAb 3h1 was obtained as a pool of four individual serum samples randomly selected from specimens sent for routine autoantibody detection at the Prince of Wales Hospital in Hong Kong; these four sera, which had a clear appearance and were negative for the
original TUBEX test and for anti-nuclear antibodies, were obtained from patients with various complaints (recurrent miscarriage, anaemia, oral ulcer and tinnitus). Some of these sera were also negative for anti-cyttoplasmic and anti-cardiolipin antibodies. Similarly, haemolytic sera were randomly selected from these specimens and were negative for anti-nuclear antibody. Whole blood was obtained from healthy laboratory workers (with consent) by venepuncture and placed in tubes containing heparin (1000 IU ml⁻¹; Mayne Pharma).

Mouse immunization. BALB/c mice were injected intraperitoneally with S. Typhi LPS (Sigma), initially with 50 μg per mouse [mixed with complete Freund’s adjuvant (Sigma)], and subsequently 2 and 4 weeks after the primary dose with 10 μg per mouse (in incomplete Freund’s adjuvant) (Wun et al., 2001). Blood was obtained from the retro-orbital plexus 1 week after the last booster, the main portion of which (100–150 μl) was placed in a tube containing 3 ml heparin, whilst the remainder was allowed to clot for use as serum. All animal manipulations followed the guidelines set out by the Animal Ethics Committee of the Chinese University of Hong Kong.

TUBEX test. The TUBEX test kit was obtained from IDL Biotech. In the existing procedure, the manufacturer’s instructions were followed except that a calibrated pipette was used to deliver an exact volume of 25 μl test sample or reagent instead of the droppers provided, which delivered a drop size of approximately 45 μl. We have also used this smaller volume previously (Rahman et al., 2007; Tam et al., 2008) and found that this did not affect the results, which depend on the concentration of the antibodies to be detected rather than their absolute quantity, i.e. when a smaller volume of test sample is used, the amounts of reagent particles used are necessarily also proportionally reduced. Conversely, it is not possible to use a larger volume of test sample than that of the reagents (to achieve a better sensitivity) in this particular protocol, as this will affect the ultimate concentrations of the particles, which have to be maintained at specific predetermined levels. The protocol is shown in Fig. 1(a). Thus, 25 μl test sample was mixed with 25 μl antigen-coated magnetic particles (Brown reagent) and stood for 2 min, before addition and mixing with 50 μl antibody-coated blue particles (Blue reagent) for another 2 min. The reaction well was then placed on a magnet stand, and the results read visually after 2 min and scored against the colour chart.

The modification of the TUBEX test was as follows (Fig. 1a). The test serum sample (25 μl) was allowed to react with the Brown reagent as above for 2 min, but more Brown reagent (50 μl) was used to accommodate possible losses during washing, and the particles were washed immediately after this initial incubation. For washing, the reaction well was stood on the magnet stand to sediment the particles. The supernatant of the reaction mixture was then carefully removed using a pipette or more conveniently a cotton-wool bud (Fig. 1c), which was inserted as far into the v-shaped well as possible without disturbing the sediment, and 50 μl glycine-buffered saline (GBS) [0.1 M glycine (Sigma) and 0.9% NaCl, final pH 8.2] was added to the sediment, without disturbing the sediment, while the reaction well was still on the magnet stand. The washing solution was carefully removed again, and 50 μl GBS buffer containing 1% BSA (Sigma) was added to the sediment; the reaction well was removed from the magnet stand and the sediment was gently mixed in the buffer. The procedure was then continued as for the existing method with the addition of 50 μl Blue reagent, and subsequent and thorough mixing of the reactants for 2 min, followed by magnetic sedimentation of the magnetic particles.

Whole blood used as the test sample in the modified procedure required a further modification in order to remove all traces of blood. This involved a second wash (Fig. 1a). Thus, after the first wash and the addition of 50 μl GBS buffer for the second time, while the reaction well was still on the magnet, the wash solution was removed again and a third lot of GBS buffer (50 μl) was added. The sediment was then suspended in the buffer (while the well was removed from the magnet stand) and used.

With both of the modified procedures, unlike the original protocol, larger volumes of test sample (and Brown reagent) relative to that of the Blue reagent can be used. This is because, following the washing, the magnetic particles (and any bound antibodies) can be resuspended to the appropriate volume (50 μl).

### RESULTS AND DISCUSSION

**Spiked clear human serum studies**

The preliminary results of our experimentation with the new procedure using GBS buffer that had been spiked with

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**TABLE 1.** The modified TUBEX procedure. (a) The modified protocol compared with the original method. Steps that differ between the two methods are indicated by shading. A ‘drop’ refers to a volume of 25 μl delivered by a graduated (Finn) pipette as used in the present study, although larger drop sizes can also be used (see Methods). (b) Results for a haemolytic serum spiked with an anti-O9 mAb (50 μg ml⁻¹) examined using the original and modified procedures. (c) Use of a cotton-wool bud to remove supernatant from the reaction mixture in the washing step.

<table>
<thead>
<tr>
<th>For clear serum</th>
<th>For clear or haemolytic serum</th>
<th>For whole blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Add 1 drop BROWN</td>
<td>1. Add 2 drops BROWN</td>
<td>1. Add 2 drops BROWN</td>
</tr>
<tr>
<td>2. Add 1 drop specimen</td>
<td>2. Add 1–8 drops specimen</td>
<td>2. Add 1–8 drops specimen</td>
</tr>
<tr>
<td>3. Mix 2 min</td>
<td>3. Mix 2 min</td>
<td>3. Mix 2 min</td>
</tr>
<tr>
<td>4. Wash, with wells placed on magnet stand:</td>
<td>4. Wash, with wells placed on magnet stand:</td>
<td>4. Wash, with wells placed on magnet stand:</td>
</tr>
<tr>
<td>- remove supernatant</td>
<td>- remove supernatant</td>
<td>- remove supernatant</td>
</tr>
<tr>
<td>- add 2 drops GBS buffer</td>
<td>- add 2 drops GBS buffer</td>
<td>- add 2 drops GBS buffer</td>
</tr>
<tr>
<td>5. Wash again, wells still placed on magnet stand:</td>
<td>5. Wash again, wells still placed on magnet stand:</td>
<td>5. Wash again, wells still placed on magnet stand:</td>
</tr>
<tr>
<td>- remove supernatant</td>
<td>- add 2 drops GBS buffer</td>
<td>- add 2 drops GBS buffer</td>
</tr>
<tr>
<td>6. Read results while wells placed on magnet stand</td>
<td>6. Read results while wells placed on magnet stand</td>
<td>6. Read results while wells placed on magnet stand</td>
</tr>
<tr>
<td>7. Mix 2 min</td>
<td>7. Mix 2 min</td>
<td>7. Mix 2 min</td>
</tr>
<tr>
<td>8. Resuspend sediment, with wells removed from magnet stand</td>
<td>8. Resuspend sediment, with wells removed from magnet stand</td>
<td>8. Resuspend sediment, with wells removed from magnet stand</td>
</tr>
<tr>
<td>9. Read results while wells placed on magnet stand</td>
<td>9. Read results while wells placed on magnet stand</td>
<td>9. Read results while wells placed on magnet stand</td>
</tr>
</tbody>
</table>
various amounts of mouse anti-O9 mAb 3h1 showed that, for the same volume of test material (25 µl), the sensitivity of detection of the spiked antibody was very similar (31.3 µg ml\(^{-1}\), taking a score of \(\leq 2\) as negative) to that of the existing (original) method (data not shown). As larger volumes of test material could be used with the modified method (but not with the original method), we investigated whether increasing the volume (but keeping the volume of Blue reagent unchanged) would increase the sensitivity of the assay. We found this indeed to be the case, with the sensitivity increasing to 15.6, 7.8 and 3.9 for 50, 100 and 200 µl volumes of test sample, respectively. This versatility is a distinct advantage of the modified procedure over the original method; in the latter, the relative volumes of the test sample and the Blue reagent must be kept fixed. These results and those of other spiked experiments in the study are representative of findings obtained from three or more experiments performed in each case.

We repeated the experiments with human serum to verify that this medium had no adverse effect on the new method. Using a clear pooled serum sample spiked with mAb 3h1 (50 µg ml\(^{-1}\)), we found that increasing the volume of test material from 25 to 50 or 100 µl increased the positive test score correspondingly (from a score of 4 to a score 6 and 8, respectively; Table 1).

### Spiked haemolytic human serum studies

We next investigated whether haemolytic human serum samples could be used with the new method. All ten samples were clearly negative when used in the modified TUBEX method but were clearly positive when spiked with 50 µg mAb 3h1 ml\(^{-1}\). The result for one of these serum samples is shown in Fig. 1(b). By comparison, when these sera were used in the original TUBEX method, the results were not readable (Fig. 1b).

### Spiked whole human blood studies

The possibility that whole blood could also be used in the new procedure was investigated using heparinized blood obtained from a healthy person, which was spiked with 25 µg mAb 3h1 ml\(^{-1}\). Detection was indeed possible (Table 1), but a second wash with GBS buffer (see Methods) was necessary to remove all traces of blood. As with buffer or serum, the TUBEX score was found to increase with increasing amounts of spiked mAb or blood volume (from a score of 6 for 100 µl spiked blood to a score of 8 for 250 µl). No reactivity was observed when blood samples from 14 other healthy individuals were similarly examined, but when these were spiked with 25 µg mAb 3h1 ml\(^{-1}\) they all gave a positive result, yielding TUBEX scores of 4–6 (data not shown).

### Immune mouse whole blood and serum studies

To verify that the modified TUBEX method worked with real specimens, we immunized mice against S. Typhi and used the blood or serum of these animals for the study, as we did not have direct access to actual samples from typhoid patients. Blood (100 µl) was examined by the modified TUBEX method and serum (25 µl) by the original method. The results obtained from the two methods were very similar (Fig. 2a). Thus, both methods detected antibodies (TUBEX scores of 3–7) in five out of eight immunized mice, with comparable reactivities, with an additional mouse (number 6) being marginally positive by the modified method; none of the five non-immunized (control) mice were reactive by either method. In four of the responder mice, the TUBEX scores were identical between the original and modified methods, but the score was slightly better with the original protocol in mouse number 7 (score of 5 vs 3). Higher reactivities were expected for the whole blood samples used in the modified method, as 100 µl blood is roughly equivalent to at least 45 µl serum, and this volume should yield a higher TUBEX score.

### Table 1. Comparison of the efficiency of detection of spiked anti-O9 mAb in different volumes of serum and whole blood between the original TUBEX method and the wash-modified method

Results are shown as TUBEX scores and are representative of three independent experiments.

<table>
<thead>
<tr>
<th>Test</th>
<th>Volume of test sample (µl)</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mAb†</td>
<td>No mAb</td>
<td>mAb*</td>
<td>No mAb</td>
<td>mAb*</td>
</tr>
<tr>
<td>Normal serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original method</td>
<td>4</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Modified method</td>
<td>4</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Normal blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modified method</td>
<td>6†</td>
<td>0†</td>
<td>8‡</td>
<td>0‡</td>
<td></td>
</tr>
</tbody>
</table>

NA, Not applicable.
* mAb 3h1 added at 25 µg ml\(^{-1}\).
† 150 µl 2 \(\times\) GBS buffer buffer for buffering.
‡ 39 µl 10 \(\times\) GBS buffer buffer.
grey bars, 25 µl month after the booster. The shaded area denotes results normally modified methods using different amounts of serum, obtained 16 days after the booster. (b) Comparison of the original and method using whole blood. Both serum and blood were obtained Comparison of the original method using serum with the modified Typhi LPS using the original and modified TUBEX procedures. (a) score than the 25 µl sample used in the original method (see below). This shortcoming may be due to the presence of red cells or other substances in whole blood, which could interfere with the mobility of the antibodies in the sample, rather than the possibility that a longer incubation period is required to complete the reaction for the larger reaction volume used (Table 1). Indeed, it is possible that if more blood (e.g. 200 µl) was used in the modified method, detection might actually be enhanced. It was not possible to verify this in the present study because of the small blood volume in the mouse, but using pooled mouse serum or clinical specimens in the future will allow investigation of this interesting prospect.

**Immune mouse serum studies using a larger volume to increase sensitivity**

An important implication of these studies is that the modified TUBEX method could be superior to the original method, even for clear serum samples. This is because (i) a greater sample volume (e.g. 50 or 100 µl) relative to the volume of Blue reagent can be used to enhance the sensitivity of the test, and (ii) the washing step, which requires minimal time and effort, reduces any background colour of the specimen and thus standardizes the specimens used. To verify this supposition, the mice used in the preceding experiments were re-bled 1 month after the last booster. The serum obtained from each animal was used in parallel in the original TUBEX method using 25 µl serum and in the modified method using twice as much serum (50 µl) (both using the same amount of Blue reagent). The results displayed in Fig. 2(b) showed that, indeed, with the larger serum volume used, the modified method was more sensitive than the original method, detecting six of the immunized mice, with TUBEX scores of 5–9, compared with scores of 4–7 using the original method for the same six animals. The remaining two mice, as well as all five non-immunized mice, were negative in both methods. Again, it will be useful to extend these studies to typhoid patients, although we already know using samples from 19 healthy individuals that normal sera (100 µl) remains unreactive in the modified method (data not shown).

**Use of the modified method to resolve borderline positives**

If the original TUBEX method is preferred for routine use, the modified method can still play a useful, supplementary role for special cases such as serum samples that are haemolytic or icteric or, as revealed in our above studies, to resolve cases that are found to be borderline positive in the original method. Indeed, it is often difficult to assess whether reactivity in the latter is an assay artefact (background noise) due to non-specific factors or cross-reactive antibodies, or whether it truly reflects the presence of low antibody levels—a dilemma relevant not only to TUBEX but also to all immunoassays, including ELISAs and the Widal test. It is possible to resolve this dilemma encountered by the original TUBEX method by repeating the test with the modified protocol using a larger volume of test serum. If the case is truly typhoidal, an increase in TUBEX score proportional to the sample size would be expected. If not, no such increase would be seen or, based on three serum samples from our routine autoimmune serum bank that had low TUBEX reactivity (scores of 3–4, most of such sera are negative), the reactivity would actually disappear in the modified method, regardless of the sample volume (data not shown). Cross-reactive antibodies, which would behave like typhoidal antibodies, are unlikely to be the cause of these non-specific reactions because the O9 antigen comprises a very small and highly unique sugar in nature.

Thus, the modified TUBEX test has several advantages over the original method: (i) it is more accurate; (ii) it allows heavily coloured serum specimens to be used that are otherwise unusable; this is important, as haemolytic sera, for example, can account for a significant percentage of a routine laboratory’s specimens; and (iii) it allows whole blood to be used as a specimen, which is particularly important if the test is to be used in the field and other point-of-care venues that do not have separation facilities for serum procurement. Together with the convenient
option of using cotton-wool buds for aspiration in the wash step, this makes the modified test an ideal diagnostic tool for use in the developing world (Mabey et al., 2004).

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REFERENCES