Optimization of Culture of *Leptospira* from Humans with Leptospirosis

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A prospective study of 989 patients with acute febrile illness was performed in northeast Thailand to define the yield of *Leptospira* from four different types of blood sample. Based on a comparison of the yields from whole blood, surface plasma, deposit from spun plasma, and clotted blood samples from 80 patients with culture-proven leptospirosis, we suggest a sampling strategy in which culture is performed using whole blood and deposit from spun plasma.

Leptospirosis is an acute febrile illness caused by pathogenic members of the genus *Leptospira*. This disease has a worldwide distribution but is most common in tropical regions (5). In a prospective observational study of undifferentiated fever in 845 patients in rural Thailand, leptospirosis was responsible for 36.9% of cases (10). The clinical features of the disease are broad ranging, but are often similar to those of other infections that commonly coexist in the tropics, such as scrub typhus, dengue fever, and malaria (10). As a result, the accuracy of a clinical diagnosis of leptospirosis is low and confirmation requires the use of laboratory tests, such as the microscopic agglutination test, which uses a panel of leptospires as antigens to detect agglutinating antibodies. The definitive diagnosis is made by the culture and isolation of *Leptospira* cells using Ellinghausen, McCullough, Johnson, and Harris (EMJH) medium. The standard method is to inoculate 1 to 5 drops (100 to 200 μl) of whole blood into EMJH medium, the basis for the small blood volume being to avoid inhibition of leptospire growth by antibodies, antibiotics, hemoglobin, or other blood component factors (2, 3). *Leptospira* survives in commercial blood culture bottles, although these samples require subculture to alternative media for bacterial isolation (4, 8). Culture is rarely used in the clinical setting because of the need for prolonged culture and the low diagnostic sensitivity, but this technique has an important role in the study of outbreaks and global epidemiology and provides a crucial pool of clinical strains for studies of pathogenesis. The microscopic agglutination test provides a broad idea of the serogroups in a given sample and is usually performed on a small blood volume in 24 h. Given the need for isolation of *Leptospira* strains and the development of strain collections associated with human disease, we undertook a study of patients presenting to hospital with a febrile illness in a tropical region where leptospirosis is endemic, to evaluate which blood sampling strategy is associated with the highest yield of *Leptospira* cells.

A prospective cohort study was conducted between May 2001 and November 2002 at Udon Thani General Hospital in northeast Thailand. The study protocol was approved by the Ethical Committee of the Ministry of Public Health, Royal Government of Thailand. Consecutive adult patients (>14 years) who presented with acute undifferentiated fever (<15 days) were recruited following informed and written consent. A detailed history and the physical examination findings were recorded on a clinical record form. A 10-ml blood sample was collected in a sterile tube containing 250 U of heparin sodium (Heparin Leo; Leo Pharma, United Kingdom) on the day of admission for *Leptospira* culture, together with a 10-ml sample of blood collected in a sterile plain tube for serology testing and culture of leptospires from clotted blood. Culture of leptospires from blood was performed using 3 ml of EMJH supplemented with 3% rabbit serum and 0.1% agarose in 5-ml sterile plastic flat-based screw-cap tubes (Sterilin; Barloworld Scientific Ltd., United Kingdom). One milliliter of blood from the heparin tube was initially separated and set aside at room temperature (25 to 30°C); this acted as reference stock which was used for further reculturing as necessary. Four sampling methods were used to isolate leptospires, in the following order. (i) For whole blood 100 μl of heparin blood was placed directly into EMJH. (ii) For surface plasma the remaining 8.9 ml of heparin blood was centrifuged at 3,000 rpm for 10 min and left undisturbed for 30 min, after which 500 μl of surface plasma was carefully removed and placed into EMJH. (iii) For the deposit from spun plasma, a further 3 ml of plasma from the heparin tube was collected and placed into a new
tube, which was centrifuged at 6,000 rpm for 3 min. The supernatant was removed to leave a 200-μl volume of deposit, which was resuspended and placed into EMJH. (iv) For blood clot samples, the clotted sample taken for serological testing was centrifuged at 3,000 rpm for 10 min and the serum removed. Three milliliters of EMJH was added to the remaining blood clot using a sterile pipette and left overnight at room temperature. The next morning, the supernatant was transferred into a new 5-ml tube.

All cultures were incubated aerobically and protected from light at room temperature (25 to 30°C) for six months; the cultures were examined weekly for three months and then every 2 to 4 weeks for a further 3 months by placing one drop of the culture onto a microscopic glass slide and observing it by dark-field microscopy at 200 magnification. Positive cultures were referred to the WHO/FAO/OIE Collaborating Center for Reference and Research on Leptospirosis, Australia, for identification using the cross-agglutinin absorption test (9).

The comparative sensitivity of each sampling method was calculated by comparing the yield of each individual method with the cumulative positive result for all four methods. Statistical analysis was performed using STATA/SE, version 9.0 for Windows (Stata Corporation, Texas) or SPSS for Windows, Release 11.0.0 (SPSS Inc.). The Cochran Q test was used to determine the homogeneity among the four methods, and the post hoc pairwise test was performed using the McNemar test. The Kaplan-Meier method was used to assess the difference in time to culture positivity.

A total of 989 patients with a clinical diagnosis of acute febrile illness were recruited during the study period. Leptospires were isolated from 83 (8.4%) patients. The median duration of fever prior to presentation was 2 days (interquartile range [IQR], 2 to 3 days), with a maximum of 5 days. The serovar determinations for cultured Leptospira cells were as follows: L. interrogans serovar Autumnalis (n = 65), L. interrogans serovar Medanensis (5), L. interrogans serovar Bataviae (3), L. interrogans serovar Pyrogenes (3), L. borgpetersenii serovar Javanica (1), L. interrogans serovar Grippotyphosa (1), and unidentified (8).

Three (4%) patients’ samples were cultured using only three of the four methods and were excluded from further analysis. The three methods used for these three patients’ samples were all positive for Leptospira. The yields of the four methods for the remaining 80 patients’ samples are shown in Table 1. There were significant differences in the yields of the four methods used (P = 0.01). The yields from whole blood, surface plasma, and deposit plasma were not significantly different from each other, but the yield of culture from clotted blood was significantly lower than the yields of these three (P = 0.03, 0.02, and 0.01, respectively). The yields were determined for combinations of sampling methods (Table 1). The combination of whole blood and deposit from spun plasma gave the highest yield, at 96.3%.

The median time to the first positive culture for the 80 patients’ samples was 21 days (IQR, 14 to 28 days). The median time to culture positivity for each method is shown in Table 1. In a Kaplan-Meier survival analysis, there was no significant difference in time to positive culture for the four methods (P = 0.08; log rank test).

When a patient’s sample was observed to be culture positive from any sample type, a repeat culture was performed on the day of positivity using 100 μl of heparinized whole blood from the reference sample stored at room temperature (thus, the median time to repeat culture was 21 days, with a range of 7 to 84 days). The purpose of this was to determine whether Leptospira survived in human blood, an observation that could be useful to studies in the field where samples cannot be cultured immediately. Among the 83 patients’ samples with one or more methods positive for leptospires, the stored sample remained positive in 52 (62.7%) cases. The median time to repeat positivity was 21 days (IQR, 14 to 28 days). The longest duration after which leptospires were recovered from heparinized blood stored at room temperature was 109 days.

Leptospiremia usually occurs within the first week of illness (7). This is consistent with our finding that the maximum duration of fever in patients who were culture positive for leptospires in their blood samples was 5 days. The predominant serovar of Leptospira in northeast Thailand was L. interrogans serovar Autumnalis, the predominant serovar cultured from small rodents in the northeast of Thailand (1). The longest duration of time to culture positivity for the primary culture was 84 days, indicating that cultures should be maintained for at least 3 months before a negative result can be reported. The current practice of using 1 to 5 drops from a plastic pipette (approximately 100 to 200 μl) represents culture of a small blood volume compared with blood culture practice for other pathogens. This could result in low diagnostic sensitivity if the number of bacterial CFU in the blood is low. In this evaluation, however, the positivities from other sample volumes were not significantly higher than those for 100 μl of heparin blood, although using combinations of methods enhanced yield. We suggest a sampling strategy in which culture is performed using whole blood and deposit from spun plasma.

### Table 1. Isolation of Leptospira cells using four sampling methods

<table>
<thead>
<tr>
<th>Sample method (n = 80)</th>
<th>Median (IQR) no. of days of fever prior to culture</th>
<th>Median (IQR) no. of days to positive culture</th>
<th>% Yield (95% confidence interval)a</th>
<th>% Yield of combinations of methods (95% confidence interval)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td>2 (2–3)</td>
<td>21 (14–28)</td>
<td>81.3 (72.5–90.0)</td>
<td>Whole blood</td>
</tr>
<tr>
<td>Surface plasma</td>
<td>2 (2–3)</td>
<td>21 (14–28)</td>
<td>78.8 (69.6–87.9)</td>
<td>Surface plasma</td>
</tr>
<tr>
<td>Deposit from spun plasma</td>
<td>2 (2–3)</td>
<td>21 (14–28)</td>
<td>82.5 (74.0–91.0)</td>
<td>Deposit plasma</td>
</tr>
<tr>
<td>Clotted blood</td>
<td>3 (2–3)</td>
<td>21 (14–34)</td>
<td>65.0 (54.3–75.7)</td>
<td>Whole blood</td>
</tr>
</tbody>
</table>

a Calculated by comparing the yield of each method with the cumulative positive result for all four methods.
b Calculated by comparing the yields of the combined methods with the cumulative positive result for all four methods.
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