Comparison of Blood Culture Methods for Recovery of Legionella pneumophila from the Blood of Guinea Pigs with Experimental Infection

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Blood was cultured from guinea pigs with experimental Legionella pneumophila serogroup 1 pneumonia, using four different methods. A 0.03-ml amount was spread onto each of several plates of buffered charcoal-yeast extract supplemented with alpha-ketoglutarate (BCYE) (direct plate); 1.5 ml each was inoculated into a BCYE agar-yeast extract broth bottle (biphasic), a pediatric Isolator tube (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.), and a glass tube containing 0.025% sodium polyanethanol sulfonate. Blood processed in the Isolator tube was plated on BCYE, as was the buffy coat blood fraction, which was obtained by centrifugation of the tube containing sodium polyanethanol sulfonate and blood. Observations were made of the number of positive cultures, the time to detection of positive cultures, and the absolute bacterial concentrations. Each system was equally sensitive in detecting bacteria. The biphasic method required 5 days for cultures to become positive, whereas the other systems required 2 to 3 days to detect all positive cultures (P = 1.3 × 10−7 by Friedman group statistic, and P < 10−5 for comparison of the biphasic and other methods). The direct plating method demonstrated the best quantitative recovery of L. pneumophila in comparison to the other methods tested (P = 2.0 × 10−5 by analysis of variance group statistic and P < 0.05 for comparison between each of the methods). Quantitative recovery by the Isolator method was intermediate between the direct plating and buffy coat methods. The biphasic and Isolator blood culture methods performed poorly in comparison to the other methods, indicating the need for caution in choosing blood culture methods for Legionella isolation.

The optimal blood culture technique for Legionella spp. has not been determined. We compared four different blood culture methods for recovery of Legionella pneumophila serogroup 1 from guinea pigs with pneumonia and bacteremia following experimental infection by intratracheal inoculation. We studied the Isolator system (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.), a biphasic culture system, direct plating of anticoagulated blood, and direct plating of the buffy coat blood fraction. (This work was presented at the 14th International Congress of Chemotherapy in Kyoto, Japan, June 1985 [abstr. no. P-55 83].)

MATERIALS AND METHODS

Animal methods. Male Hartley strain guinea pigs weighing 300 to 400 g were used as described previously (5). Intratracheal inoculation of the animals was based on the methods of Winn and colleagues (14). The trachea was surgically exposed by using an aseptic technique. A total of 6.6 × 10⁶ CFU of a clinical isolate of L. pneumophila serogroup 1 (Wadsworth isolate W82-063A) was suspended in 0.3 ml of normal saline and injected into the trachea with a 1-ml syringe equipped with a 25-gauge needle. The incision was closed with steel clips. Animals were observed several times daily for evidence of illness. Two to three days after inoculation, animals were exsanguinated by cardiac puncture under direct visualization, using a heparin-wetted syringe; ketamine-xylazine general anesthesia with local lidocaine anesthesia was used (5).

Recovery efficiency with a seeded sample. We first determined if the strain of L. pneumophila used in the animal model was inhibited by components of the Isolator system. A clinical isolate of L. pneumophila serogroup 1 (strain W82-063A) was grown until late log phase in 10 ml of buffered yeast extract broth shaken at 35°C overnight and then centrifuged at 3,000 × g for 20 min. The pellet was washed once with 10 ml of normal saline, centrifuged under the same conditions, and then suspended in a portion of the supernatant. The bacterial concentration was determined by plating serial 10-fold dilutions on buffered charcoal-yeast extract (BCYE). A 1.5-ml portion of serial 10-fold dilutions of the bacterial suspension in sterile normal saline was added to pediatric Isolator tubes. Each tube was mixed by inversion and incubated for 30 min at room temperature, and 0.1-ml portions of the contents were plated to each of five BCYE plates. The pediatric Isolator tubes were not centrifuged in accordance with the manufacturer's instructions. Colonies were enumerated after 72 h of incubation of the plates in a 35°C air incubator (90% relative humidity).

Blood culture protocol. Blood from the infected guinea pigs was distributed in duplicate into each of the four test method.
were identified as *L. pneumophila* on the basis of colonial and microscopic morphology, growth tests, Gram stain, and direct immunofluorescence examination (4).

**Statistical analysis.** Quantitation of bacteria in each system was normalized to 1.0 ml of blood. The Friedman group statistic was used for comparison between the mean number of days to first positive blood culture for each method (2). Descriptive statistics of quantitative colony counts were derived by using an assumption of a Poisson distribution (13). Computer-aided analysis of variance was used for comparison of the mean *L. pneumophila* concentrations recovered by each method (13) (Stats Plus; Human Systems Dynamics, Northridge, Calif.). A two-tailed Mann-Whitney test was used to compare quantitative plating results and direct plating results (2).

**RESULTS**

In the preliminary study, there was no significant inhibition of broth-grown *L. pneumophila* suspended in saline and processed by the Isolator method. Colony counts of *L. pneumophila* suspended in saline were $4.5 \times 10^8$ CFU/ml and counts of the suspension first processed in the Isolator were $4.2 \times 10^9$ CFU/ml. Fifteen animals were used for all four blood culture methods. Gross examination of the lungs of all animals at necropsy revealed acute, bilateral hemorrhagic consolidating pneumonia. Lung impressions stamped on BCYE agar plates were positive for *L. pneumophila* serogroup 1 from all animals. The mean number of CFU per milliliter of blood for three different animals determined by direct quantitative plating was $1.3 \times 10^8$, with a range of $0.1 \times 10^8$ to $2.4 \times 10^8$ and a standard error of $\pm 0.5 \times 10^4$. As a group, all three blood samples processed by the quantitative plating method had significantly greater concentrations of *L. pneumophila* detected in the blood than did the group of 15 blood samples processed by direct plating of 0.03 ml of blood ($P < 0.01$ by the Mann-Whitney test).

Positive blood cultures for *L. pneumophila* serogroup 1 were observed with each method for all 15 animals. Numbers of *L. pneumophila* bacteria detected were markedly different between the three methods that could be quantitated. Direct plating yielded the greatest number of bacteria, whereas plating of blood buffy coat produced the lowest number ($P < 2 \times 10^{-5}$ for group comparison) (Table 1). There were no differences in culture noted when plates were held for as long as 5 days. There were significant differences noted in the time to first detection of a positive sample, with the bipheric system requiring 5 days of incubation and the other systems requiring 2 to 3 days of incubation (Fig. 2). More contaminants were observed on the BCYE plates after processing by the Isolator method compared with the other

<table>
<thead>
<tr>
<th>Test method</th>
<th>No. of culture-positive animals</th>
<th>Mean no. of days to 1st positive*</th>
<th>Range of days to 1st positive</th>
<th>Mean CFU/ml (±SEM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biphasic</td>
<td>15/15</td>
<td>4.2</td>
<td>3-5</td>
<td>840 ± 260</td>
</tr>
<tr>
<td>Isolator</td>
<td>15/15</td>
<td>2</td>
<td>2-3</td>
<td>260 ± 180</td>
</tr>
<tr>
<td>Buffy coat</td>
<td>15/15</td>
<td>2.1</td>
<td>2-3</td>
<td>3,200 ± 1,700</td>
</tr>
<tr>
<td>Direct plate</td>
<td>15/15</td>
<td>2</td>
<td>2-3</td>
<td>3,200 ± 1,700</td>
</tr>
</tbody>
</table>

* $P = 1.3 \times 10^{-7}$ by Friedman (group) statistic and $P < 10^{-5}$ for comparison of bipheric system and other systems.

* $P = 2.9 \times 10^{-7}$ by analysis of variance (group) statistic and $P < 0.05$ for comparison between each of the methods.
systems evaluated, but this did not affect the statistical significance of the results.

**DISCUSSION**

Our results show that the previously used method of biphasic medium isolation of *L. pneumophila* from blood is a suboptimal one (6, 11). Conversely, direct plating of heparinized blood on a small volume per plate may significantly increase yield and decrease detection time compared with the biphasic system. Despite the known phagocytosis of *L. pneumophila* by mononuclear cells, culture of lysed leukocytes did not enhance culture sensitivity (7).

Several factors may have been responsible for the differences noted between culture methods. Most probable is inhibition of *L. pneumophila* by blood components; this is supported by the 10-fold-lower bacterial counts for the best method (direct plating) than for quantitative counts performed by serial dilution in Mueller-Hinton broth, in which inhibitors may have been diluted beyond their active concentrations. Prior studies in this laboratory have shown no inhibition of broth-grown *L. pneumophila* by BCYE or by the broth and solid medium used in the biphasic culture system (P. Edelstein, unpublished data). On the other hand, growth inhibition of *L. pneumophila* by some animal and human tissues has been observed, especially by lung and spleen (8; Edelstein, unpublished observation). It was for this reason that small blood volumes were used in direct plating of blood. It is possible that direct plating of serial dilutions from Mueller-Hinton broth or direct plating of 0.03 ml of lysed or buffy coat blood would improve recovery efficiency. Since we did not study this, nor interaction of our strain with individual blood components, this is only speculative. However, it does seem unlikely that the Isolator system components themselves were inhibitory based on our preliminary study.

Extrapolation of these results directly to detection of human bacteremia caused by *L. pneumophila* must be tempered by our use of an animal model to produce bacteremia. Two factors must be considered in this regard, the bacterial concentration in blood and the related possibility that some of our blood may have inadvertently contained *L. pneumophila* bacteria present in a pericardial abscess. Although the true concentration of *L. pneumophila* in blood taken from patients with Legionnaires' diseases is unknown, it probably is in the range of 10 to 700 CFU/ml; this was far exceeded by the concentration of organisms found in guinea pigs (12). This very high concentration made bacterial detection easy using small volumes of blood. It might be very difficult, or at least tedious, to detect fewer than 50 to 100 CFU of *L. pneumophila* per ml by direct plating of small blood volumes. For this reason, methods which concentrate or amplify bacteria in blood samples, or which do both, are presumably preferable. The other consideration is the possibility that we sampled blood and pericardial abscess contents. This seems unlikely since we observed no gross evidence of pericarditis in any of our animals and were very careful to directly visualize needle entry and exit into the heart to avoid contamination with other tissues. If occult contamination of the sample occurred with nonhematogenous *L. pneumophila*, then differential numbers of phagocytes and other factors might have influenced these results.

It is also possible that the strain of *L. pneumophila* we used behaved in an atypical fashion and that study of other strains would change our conclusions. For this reason, and because of the high-grade bacteremia observed in our experimental model, these results must be regarded as tentative and in need of confirmation by clinical studies.

**ACKNOWLEDGMENT**

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**LITERATURE CITED**