Novel Plate Culture Method To Improve the Microbiological Diagnosis of Peritonitis in Patients on Continuous Ambulatory Peritoneal Dialysis

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A novel economical plate culture technique incorporating Tween 80 was used for the isolation of organisms from dialysis effluent in cases of continuous ambulatory peritoneal dialysis peritonitis. It was found to be convenient for routine laboratory use and increased the yield of positive plate cultures in specimens without antibiotics from 53 to 75% ($P < 0.01$) and in specimens containing antibiotics from 24 to 38% ($P < 0.05$). It allowed for the identification and susceptibility testing of isolates 24 h earlier than did broth culturing and a more ready appreciation of mixed cultures and contaminants.

Bacterial peritonitis continues to be a common problem in the treatment of renal failure by continuous ambulatory peritoneal dialysis (CAPD). Broth culturing is the single most sensitive culture technique for the diagnosis of this condition (6, 11, 16), but it is conventional and good microbiological practice to perform a quantitative plate culture simultaneously. This practice allows early recognition of contaminates and mixed cultures and more rapid isolation of single colonies for identification and susceptibility testing. At best, however, conventional plate culture techniques have a sensitivity of about 50% for CAPD peritonitis (11, 16). We and others have described methods for improving the sensitivity of plate cultures with nonionic surfactants (6, 9, 18), pour plates (5), total volume cultures (4), and filtration techniques (13, 19). Each of these methods is, however, relatively time-consuming and/or subject to contamination. In this paper we present results obtained with lysis-centrifugation and nonionic surfactants and describe a method suitable for routine use in the diagnosis of CAPD peritonitis.

**MATERIALS AND METHODS**

The study was carried out from October 1985 to May 1986 in the routine diagnostic bacteriology laboratory of the Queen Elizabeth Hospital, Birmingham, United Kingdom, which serves a large renal unit where over 140 patients are on CAPD. Specimens of dialysis effluent from these patients are sent to the laboratory if peritonitis is suspected. It is routine to send fluid from the first cloudy bag. Specimens are also sent to monitor responses to antibiotic therapy in cases of peritonitis. During the study period, if dialysis effluent was to be sent for analysis, 160 ml was aspirated aseptically from the dialysis bag by nursing staff on the dialysis unit using 60-ml sterile syringes (Sterilin Ltd., Feltham, Middlesex, United Kingdom). One hundred milliliters was immediately placed in a sterile container and sent to the laboratory during normal working hours or stored at 4°C until the following morning if collected after 5 p.m. Sixty milliliters of effluent was aseptically inoculated into 20 ml of four-times-normal-strength no. 2 nutrient broth (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) as previously described (7) and incubated immediately at 37°C. On arrival in the laboratory cell counts were performed on all fluids. Any fluid with >100 polymorphs per mm$^3$ was considered to have come from a case of peritonitis (6) and was entered into the study. A total of 114 consecutive such fluids were studied by the following methods. Aliquots (15 ml) of effluent were separated aseptically into six 20-ml plastic universal containers (Sterilin) and processed as follows. To the first aliquot at room temperature was added 2 drops (Pasteur pipette) of Triton X-100 (BDH, Poole, Dorset, United Kingdom) for 30 min prior to centrifugation at 3,000 rpm (1,486 × g) for 10 min and culturing of the entire deposit on 7% horse blood agar (Difco Ltd., East Molesley, Surrey, United Kingdom) containing Columbia agar base (Oxoid). The other five aliquots were first centrifuged at 3,000 rpm (1,466 × g) for 10 min, and then the entire centrifuged deposit was processed in different ways. The deposit from the second aliquot was cultured directly on 2% Tween 80 agar containing 7% horse blood. Five drops of sterile H$_2$O from a sterile Pasteur pipette was added to the deposit from the third aliquot and left for 10 min at room temperature before recentrifugation and culturing of the entire deposit on 7% horse blood agar. For the fourth aliquot, the entire centrifuged deposit was lysed with 1 drop of Triton X-100 at room temperature for 5 min and cultured on 7% horse blood agar. The deposit from the fifth aliquot was cultured directly on 7% horse blood agar. Incubation for these five methods was done in 5% CO$_2$ at 37°C for up to 72 h. The deposit from the sixth aliquot was cultured directly on cysteine-lactose electrolyte-deficient agar (Oxoid) and incubated in air at 37°C for up to 72 h.

The 60 ml of effluent inoculated into four-times-normal-strength nutrient broth was incubated at 37°C for 9 days, examined daily, and subcultured and Gram stained if turbid. Routine subculturing was done at 24 h and 9 days.

In addition, an antibiotic detection plate (15) was set up for all but one of the fluids as described by Pelling (14) to detect antibacterial substances in urine, but with the following modifications. A diluted overnight broth culture or spore suspension of Bacillus subtilis NCTC 8236 was flooded onto Direct Sensitest Agar plates (Oxoid), the excess was pipetted off, and the plates were dried and stored at 4°C for up to 14 days. For use, a well was cut with a no. 4 cork borer, 100 µl of effluent was added, and the plate was incubated overnight at 37°C. Any inhibition of growth was recorded as

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the presence of antibacterial substances. This method has been in use for several years at a neighboring hospital to monitor antibacterial activity in urine specimens submitted for culturing (D. Healing, personal communication). The strain of *B. subtilis* used is sensitive to all of the antibiotics knowingly administered to our patients, i.e., aminoglycosides, β-lactams, and vancomycin, and *B. subtilis* is a recommended assay organism for some aminoglycosides and vancomycin (Difco Manual, 10th ed., p. 80–82, 1984).

Triton X-100 and Tween 80 (BDH) are 100% pure, self-sterilizing, nonionic surfactant agents. Tween 80 (2%; 20 ml) incorporating Columbia agar base (39.0 g; Oxoid), distilled water (910 ml), and horse blood (7%; 70 ml) (Difco) was prepared by the following method. The agar was mixed, and the Tween 80 was added. The mixture was autoclaved at 121°C for 15 min and allowed to cool, the horse blood was added, and the plates were poured. Plates were kept at 4°C for up to 4 weeks.

Patients’ charts were reviewed to confirm the presence or absence of peritonitis. A relapse was defined as the same organism isolated for a second time within a 3-week period. If the time period was longer or the organism was different from the first isolate, it was considered a separate episode of peritonitis. Statistical analysis was done by approximate contingency table tests based on the chi-square test (8). The tables consisted of positive and negative results for each of the six methods, but only the positive results are shown (see Tables 1 and 2). Quantification of growth for calculating growth indices was as follows: 0, no growth; 1, less than five colonies in the primary inoculum; 2, more than five colonies in the primary inoculum; 3, growth in the second inoculum; and 4, growth in the third or fourth inoculum. Plates were inoculated by standard methods (2) involving placing of the deposit in a well (the primary inoculum) with a sterile Pasteur pipette and spreading of the primary inoculum, with flaming of the loop between each step, to produce second, third, and fourth inocula.

Isolates were identified by the methods of Cowan (1), testing with API test strips (API Systems, SA, Montalieu, Vercieu, France), phage typing (3) (for *Staphylococcus aureus*), and biotyping (10) (for coagulase-negative staphylococci). When similar isolates were obtained from broth and plate cultures for the same patient, a combination of these methods and antibiograms confirmed isolates as identical or different.

### RESULTS

A total of 114 fluids were analyzed; 93 were from separate episodes of peritonitis in 77 patients, and 52 contained no antibiotics. In addition, there were repeat specimens on 21 occasions, 6 of these from apparent relapses; all of these specimens contained antibiotics. All specimens came from patients either at the first occurrence of an episode of peritonitis or receiving antibiotic therapy for such an episode.

Table 1 shows the isolation frequencies for the different culture methods. There were 74 separate isolates from 71 specimens, including 39 strains of coagulase-negative staphylococci and 10 of *S. aureus*. Three patients had apparent mixed infections. The other gram-positive organisms were five diphtheroids, one *Streptococcus faecalis*, four alphahemolytic streptococci, one nonhemolytic streptococcus, and three *Bacillus* spp. The gram-negative organisms were two *Pseudomonas aeruginosa*, two *Serratia* spp., two *Acinetobacter* spp., two *Escherichia coli*, one *Moraxella* sp., and one *Neisseria* sp. All but three isolates grew within 18 h of plating the specimens.

Methods 1, 2, and 4, which make use of a surfactant, yielded significantly higher isolation frequencies than did methods 3, 5, and 6 (*P* values of <0.001 to <0.025; Table 1) for coagulase-negative staphylococci, total gram-positive organisms, and total positive cultures, and *P* values were <0.05 when individual methods were compared for total positive cultures. The novel plate methods were as good for the isolation of coagulase-negative staphylococci and *S. aureus* as was broth culturing.

Figure 1 shows the distribution of growth indices for each of the six plate culture methods in the 71 culture-positive specimens. Again, the novel plate methods (1, 2, and 4) yielded significantly better growth indices both as a group (*P* < 0.001) and individually (*P* < 0.001 to *P* < 0.025). The isolation rates were strikingly different depending on the presence or absence of antibacterial activity (Table 2), and the differences as a group between methods 1, 2, and 4 and methods 3, 5, and 6 remained significant in the presence or absence of antibiotics (*P* < 0.05 and *P* < 0.01, respectively). In the absence of antibiotics, broth culturing yielded isolates in 88% of specimens, while in combination with any one of the novel plate culture methods it yielded positive culture results in at least 48 of the 52 (92%) fluids. The novel plate

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**TABLE 1. Number of isolates detected by each culture method in the 114 CAPD effluents**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>No. of isolates detected by culture method:</th>
<th>Total no. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Coagulase-negative staphylococci</td>
<td>38</td>
<td>39</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Other gram-positive organisms</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Total gram-positive organisms</td>
<td>58</td>
<td>57</td>
</tr>
<tr>
<td>Gram-negative organisms</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total positive</td>
<td>66</td>
<td>65</td>
</tr>
</tbody>
</table>

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* a * P < 0.025.

* b * P < 0.001 for methods 1, 2, and 4 versus methods 3, 5, and 6.

* c * P < 0.01 for method 2 versus method 5.

* d * P < 0.05 for method 2 versus method 6.
culture methods increased the positive plate culture rate from a mean of 53.7 to 76.3% when no antibacterial activity was detected ($P < 0.01$) and from 24.3 to 36.6% in specimens containing antibacterial activity ($P < 0.05$).

**DISCUSSION**

All three plate culture methods that used the surfactant materials Triton X-100 and Tween 80 significantly increased the rate of positive cultures (Table 1) and the growth indices (Fig. 1) as compared with conventional plate culture techniques. Two possible mechanisms for these results are lysis of polymorphs, liberating intracellular bacteria before they are killed, and separation of bacteria by a reduction in surface tension, thus allowing more CFUs to be spread on the plates.

We agree with Taylor et al. (18) that the separation of bacteria is likely to be  the less important mechanism in view of the success of the Tween 80 agar in increasing the number of CFUs per specimen when exposure to the surfactant occurred after plate inoculation. Our attempts at lysis with sterile water (method 3) were unsuccessful, suggesting that a reduction in surface tension between organisms may be the more important mechanism, but we may have used inadequate volumes of water and lengths of exposure to the sample to yield optimum lysis. Kleiman et al. (9) maintain osmotic lysis of leukocytes to be superior to surfactant agents in this context. Inactivation of antibacterial substances during lysis-centrifugation has also been suggested as a possible mechanism of action (17), although in our study a beneficial effect was maintained even in specimens in which no antibacterial substances were demonstrated (Table 2). Taylor et al. (18) advocate prior sonication of the sample in addition to exposure to the surfactant saponin. While we agree that sonication is effective in increasing CFUs (unpublished data), we feel that this is an impractical procedure for a routine diagnostic laboratory because it is particularly liable to contamination. Kleiman et al. (9) note that MacConkey agar is generally superior to blood agar in yielding CFUs and attribute this fact to the surfactant properties of bile: Taylor et al. (18) have confirmed this. In our study, cysteine-lactose electrolyte-deficient agar also appeared to be superior to blood agar, but the difference was not significant ($P < 0.05$).

Other recently described attempts to improve culture techniques for the diagnosis of CAPD peritonitis used enrichment broths (4, 13, 16). They tend to be prone to contamination and make it difficult to determine if this has occurred, as growth cannot be quantified. Males et al. (13) found conventional culturing of the centrifuged deposit from 10 ml to be as sensitive as enrichment or filtration, but the number of specimens studied was small and the deposit from 10 ml was cultured on MacConkey agar, which may have increased yields because of its surfactant properties. Some workers have used filtration (13, 16), but in our experience and that of others (9, 13), filters too often clog to be of much use.

To improve on conventional culture techniques, our Tween 80 plate method and the saponin-containing blood agar of Taylor et al. (18) appear to be the most convenient for routine use. The advantages are clear-cut (6, 9, 12, 17, 18). These include an increase in the positive plate culture rate and increased growth indices, providing more rapid isolation of individual colonies. In all cases in which isolates were also obtained in broth, identification and susceptibility testing could be carried out 24 h earlier by the novel plate culture methods. They also allow for a more ready appreciation of contaminants and mixed cultures than does broth culturing, the former being a common problem in the examination of cultures from CAPD peritonitis, in which the common pathogens are also skin flora. If a blood agar culture is also included, then the presence of intracellular, and presumably pathogenic, bacteria can easily be deduced by the difference

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**TABLE 2. Cultures found positive by different methods**

<table>
<thead>
<tr>
<th>Antibacterial activity in specimen</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Broth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present ($n = 61$)*</td>
<td>22 (36)</td>
<td>23 (38)</td>
<td>13 (21)</td>
<td>22 (36)</td>
<td>15 (24)</td>
<td>17 (28)</td>
<td>22 (36)</td>
</tr>
<tr>
<td>Absent ($n = 52$)*</td>
<td>41 (79)</td>
<td>39 (75)</td>
<td>26 (50)</td>
<td>39 (75)</td>
<td>26 (50)</td>
<td>32 (61)</td>
<td>46 (88)</td>
</tr>
</tbody>
</table>

* Antibiotic activity was tested in 113 of the 114 fluids.
* $P < 0.05$ for methods 1, 2, and 4 versus methods 3, 5, and 6.
* $P < 0.01$ for methods 1, 2, and 4 versus methods 3, 5, and 6.
in growth indices (18), so added significance is given to the culture. Finally, the use of a Tween 80 plate in addition to broth culturing should provide closer to a 100% rate of isolation of pathogens than does broth culturing alone.

Tween 80, Triton X-100 and other nonionic surfactants are being used increasingly in lysis-centrifugation methods to increase the yield of blood culture techniques. These agents are, however, toxic for bacteria in high concentrations, and further work is needed to assess the optimum type and concentration of surfactant to be incorporated in a culture medium to provide rapid cell lysis without toxicity for bacteria. The present medium is not satisfactory for the isolation of nonhemolytic streptococci and Neisseria spp. (unpublished observation), and this fact is partly confirmed by the relatively poor isolation rate for other gram-positive organisms (Table 1). Our previous studies with Triton X-100 (6) showed that the isolation rates for gram-negative bacteria were improved more than those for gram-positive bacteria, but in this study there were insufficient gram-negative isolates to comment on this.

Further work is in progress to identify the most satisfactory medium incorporating a nonionic surfactant for the culturing of CAPD effluent. Consideration should also be given to the use of this technique for routine culturing of other fluids with a high concentration of leukocytes, such as cerebrospinal, joint, ascitic, and pleural fluids.

ACKNOWLEDGMENTS

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LITERATURE CITED