and the intracystic body at the same time. Trophozoites can also be shown by Giemsa staining.

Kim et al,5 double stained P carinii on impression smears of rat lungs with methenamine silver and polychrome methylene blue and stated that these paren-thesis like structures seemed to be part of the cyst wall or at least closely related to the cyst wall. According to our observations, these structures can be mainly seen in empty cysts but sometimes in mature cysts that contain the intracystic bodies. These structures may correspond to internally thickened parts of the cyst walls. We noticed that the cyst wall of some cysts containing intracystic bodies was not stained with Gomori’s stain. Although the reason for this was not clear, one possibility is that they are thin walled pneumocysts containing daughter cells, as Vossen et al6 indicated.

It is well known that the Gomori stain staining technique is hard work: the double staining method proposed here is no less so. As a next step, modifications to speed up Gomori staining should be considered for use.7–11

This study was supported by Grant-in-Aid for Scientific Research (No 58480170) from the Ministry of Education, Science, and Culture, Japan; contribution No 555 from the Department of Medical Zoology, Kyoto Prefectural University of Medicine. I sincerely thank Professor Yukio Yoshida, the director of the Department of Medical Zoology, Kyoto Prefectural University of Medicine, Kyoto, Japan, for his interest, guidance, and encouragement throughout this study and his critical reading of this manuscript.

New method for typing coagulase negative staphylococci

J R STEPHENSON, S TABAQCHALI  From the Department of Medical Microbiology, St Bartholomew’s Hospital Medical College, London

Coagulase negative staphylococci are found as pathogens in an increasing number of infections, usually associated with prosthetic implants and catheters.1 The importance of isolates of these bacteria is often uncertain because they are also common contaminants. The interpretation of repeated isolates depends on a suitable typing system. Several methods have been used, including biotyping,2 3 antibiograms,4 phage typing,5 plasmid profile and restriction endonuclease analysis.6 No one method, however, has proved ideal, and often a combination of methods is necessary for epidemiological studies.

We developed a new typing system based on sodium dodecyl sulphate polyacrylamide gel electrophoresis of 35S-methionine labelled bacteria (radio-PAGE). This method was compared with a combination of biotyping and antibiograms using a series of coagulase negative staphylococci isolated from patients with peritonitis who were being treated with continuous ambulatory peritoneal dialysis (CAPD).

Methods

SOURCE OF STRAINS
Forty isolates of coagulase negative staphylococci were obtained from 38 episodes of acute peritonitis from eight patients who were being treated with CAPD.

MICROBIOLOGICAL METHODS
Coagulase negative staphylococci were isolated and
identified by standard laboratory techniques. Isolates were stored at \(-20^\circ\text{C}\) in 2 ml volumes of glycerol nutrient broth (containing 1-6 ml Oxoid nutrient broth No 2 and 0-4 ml of glycerol). Biotyping was performed using the API Staph (API System, La Balmes Les Grottes, France) reading the tests at the times suggested by Marples and Richardson.\(^7\) Anti-biograms were performed by the Stokes comparative method,\(^8\) testing penicillin, erythromycin, gentamicin, fusidic acid and methicillin. Results in the moderately resistant category were recorded as resistant.

**RADIOPAGE METHOD**

Strains were subcultured from storage on to horse blood agar (Gibco) and incubated overnight at 37°C. Portions of several colonies of each strain of coagulase negative staphylococcus were inoculated into 50\,\mu l volumes of methionine assay medium (Difco) containing 5 \mu Ci (185 kBq) of \(^{35}\)S-methionine (Amersham International) until the medium appeared slightly turbid. The samples were incubated at 37°C in air for three hours, and subcultures for purity checks were then made. Enzymatic digestion of the staphylococcal cell wall was achieved by the addition of 50 \mu l volumes of lysothaphin solution containing 400 \mu g/ml of lysothaphin (Sigma) in 10 mM Tris buffer followed by reincubation at 37°C for 45 minutes. After this incubation 100 \mu l volumes of double strength sample buffer\(^9\) were added, and the soluble proteins were dissociated by immersing the samples for three minutes in boiling water.

SDS-PAGE was performed according to the method of Laemmli,\(^9\) with a 3% by weight (30 g/l) of acrylamide stacking gel and a 10% (100 g/l) resolving gel of 0-75 mm thickness. On each gel 12 samples and one \(^{14}\)C methylated protein mixture aliquot (Amersham International) were loaded in 15\,\mu l volumes. Electrophoresis was carried out at constant current using 10 mA/gel for stacking and continued at 15 mA/gel throughout the resolving gel. The gels were fixed overnight in an aqueous solution containing 200 ml of glacial acetic acid and 200 ml of propan-2-ol in each litre, and then vacuum dried onto filter paper. Autoradiographs were developed after 24 hours' exposure. Comparison of labelled protein profiles was performed visually after cutting the autoradiographs into strips of two lanes each.

All 40 isolates were typed by radioPAGE in one batch, and this was repeated in a second batch.

**Results**

Pairs of isolates were constructed by taking each of the 40 isolates in turn and combining them with each of the other 39 isolates, giving a total of 780 unique pairs of isolates. The radiolabelled protein profile of one isolate in a pair was compared visually with the profile from the other isolate to obtain the radio-PAGE typing results. Isolates were recorded as being different either if one strain had one or more radiolabelled protein bands of different molecular weight, or if one strain had two or more bands of the same molecular weight, but with noticeable differences in intensity. Figs 1 and 2 show examples of the autoradiographs.

The results of the API Staph and anti-biogram were combined for each isolate giving an "extended biotype." The isolates were analysed using the same 780 pairs. One extended biotype was recorded as different from another if there were two or more differences in the combined individual biochemical tests and anti-biogram. This criterion was chosen to give optimum discrimination while retaining acceptable technical reproducibility.

The 780 pairs were analysed in two groups. The first group consisted of the 680 pairs, in which each isolate in a pair came from a different patient. Each pair in this group is likely to contain different strains, because most episodes of peritonitis developed while the patient was at home and so cross infection with the same strain should be uncommon. Analysis of these 680 pairs should measure the discrimination of each typing system.

The second group contained the remaining 100 pairs, in which both isolates in a pair came from the same patient. These pairs would contain identical isolates if there was a relapsing infection or if there had been reinfection by the same strain. Other strains would be different due to reinfections with different strains. The proportion of these 100 pairs that are different or the same is uncertain on epidemiological grounds, but comparison of radioPAGE with biotyping should give a measure of their relative reproducibilities.

**TYPABILITY**

All strains gave a profile on the API Staph, and only three isolates were sensitive to all five antibiotics in the anti-biogram.

Each isolate gave a distinct radiolabelled protein profile with radioPAGE typing.

**DISCRIMINATION**

Analysis of the 680 pairs of isolates where each isolate comes from a separate patient shows that in 655 of these pairs one isolate had a different extended biotype from the other. Using radioPAGE, 665 pairs contained strains of different radiolabelled protein profiles, and this included all 655 pairs with different extended biotypes. The additional 10 pairs concerned only two patients. The radioPAGE profiles differed by at least four major bands with several minor
Technical methods

Fig 1  Autoradiograph of coagulase negative staphylococci isolated from peritoneal fluids from patient A (lanes 1–10) and patient B (lanes 11 and 12).

<table>
<thead>
<tr>
<th>Lane</th>
<th>Date</th>
<th>RadioPAGE type</th>
<th>Lane</th>
<th>Date</th>
<th>RadioPAGE type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14 December</td>
<td>A1</td>
<td>7</td>
<td>7 June</td>
<td>A2</td>
</tr>
<tr>
<td>2</td>
<td>29 December</td>
<td>A1</td>
<td>8</td>
<td>11 June</td>
<td>A2</td>
</tr>
<tr>
<td>3</td>
<td>13 January</td>
<td>A1</td>
<td>9</td>
<td>11 June</td>
<td>A2</td>
</tr>
<tr>
<td>4</td>
<td>13 January</td>
<td>A1</td>
<td>10</td>
<td>22 July</td>
<td>A2</td>
</tr>
<tr>
<td>5</td>
<td>17 May</td>
<td>A2</td>
<td>11</td>
<td>14 November</td>
<td>B1</td>
</tr>
<tr>
<td>6</td>
<td>18 May</td>
<td>A2</td>
<td>12</td>
<td>7 August</td>
<td>B2</td>
</tr>
</tbody>
</table>

MWM = $^{14}$C molecular weight marker: 200, 93, 69, 46, 30 and 14 kDaltons.

differences in intensity in all these 10 pairs having the same biotype.

REPRODUCIBILITY

The remaining 100 pairs of isolates, where each isolate in one pair came from the same patient, were analysed. The extended biotyping showed 47 pairs of indistinguishable strains, whereas radioPAGE identified 43 pairs with the same radiolabelled protein profile. Three of the additional four pairs found to be different by radioPAGE were constructed by pairing D2 with the three isolates of D1 shown in fig 2. The strain D2 is typed as different from D1 because a band of slightly lower molecular weight than the 200 kDa marker is found in D1, but is absent in D2. D2 has a different band of molecular weight slightly larger than the 200 kDa marker. These differences were found in both batches of radioPAGE. The fourth pair of same biotype but different radioPAGE profile was distinguished by similar findings.
Technical methods

**Fig 2** Autoradiograph of coagulase negative staphylococci isolated from peritoneal fluids from patient C (lanes 1–5) and patient D (lanes 6–9).

<table>
<thead>
<tr>
<th>Lane</th>
<th>Date</th>
<th>RadioPAGE type</th>
<th>Lane</th>
<th>Date</th>
<th>RadioPAGE type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13 January</td>
<td>C1</td>
<td>2</td>
<td>27 March</td>
<td>C1</td>
</tr>
<tr>
<td>2</td>
<td>29 April</td>
<td>C2</td>
<td>3</td>
<td>14 July</td>
<td>C3</td>
</tr>
<tr>
<td>4</td>
<td>14 July</td>
<td>C3</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5 May</td>
<td>D1</td>
<td>7</td>
<td>30 May</td>
<td>D1</td>
</tr>
<tr>
<td>8</td>
<td>11 August</td>
<td>D1</td>
<td>9</td>
<td>6 September</td>
<td>D2</td>
</tr>
</tbody>
</table>

**MW** = $^{14}$C molecular weight marker: 200, 93, 69, 46 and 30 kDaltons.
Discussion

The two techniques that are readily available in routine diagnostic laboratories for typing coagulase-negative staphylococci are antibiograms and biotyping. Even with a large range of antibiotics, discrimination by antibiogram alone is not good. Biotyping using some commercially available micromethods with a small number of tests may identify only a few commonly occurring biotypes. The API Staph with 20 biochemical tests gives better discrimination and has been compared with conventional biotyping. Reproducibility is not ideal with either antibiograms or biotyping due to technical problems and possible transposon interference. Typability is good with both techniques. Phage typing may give suitable discrimination and reproducibility, but low typability is a major problem.

Plasmid profile analysis seem to be a promising technique for distinguishing infecting from non-infecting isolates of Staphylococcus epidermidis from blood cultures. This typing system, however, has not been fully evaluated and may require further steps of purification and restriction enzyme analysis to confirm the identity of plasmid bands.

Polyacrylamide gel electrophoresis of "cold" bacterial proteins has been used in taxonomic studies. Protein purification and careful standardisation of protein quantity, however, are necessary to obtain optimal staining of protein bands. Radio PAGE is a more robust technique that does not require these additional stages to give distinct bands. Radio PAGE was first described for typing isolates of Clostridium difficile and has been extended in this study to type coagulase negative staphylococci. This method requires electrophoretic apparatus similar to that used for plasmid analysis, but facilities to handle radioisotopes are also needed.

This preliminary study indicates that radio PAGE gives excellent typability. The apparently increased discrimination with radio PAGE may only reflect reduced reproducibility compared with that of extended biotyping. In the first group analysed, where the 680 pairs of isolates came from different patients, the 10 pairs indistinguishable by biotyping that were found to be different by radio PAGE had several differences between their profiles in each pair, similar to differences shown between separate strains in this study. This cannot be ruled out, but taken together, it seems likely that there is some slightly increased discrimination with radio PAGE in this group.

In the second group, however, where pairs of isolates were from the same patient, the extra four pairs that were distinguishable by radio PAGE had very similar profiles with the only difference in three of these pairs being the position of one high molecular weight band (fig 2). This difference may reflect too stringent criteria for discrimination, because most other pairs of isolates found to be different by both biotyping and radio PAGE had several different bands in their radio PAGE profiles (fig 1). There is no additional epidemiological evidence available in this group because the proportion of infections caused by new strains is unknown. The total number of discordant pairs by the two methods is small, and overall, the discrimination by radio PAGE is comparable with that obtained with the extended biotyping method. Reproducibility also seems to be comparable with that of extended biotyping, but it is optimum with radio PAGE when isolates are labelled and run in the same batch, and this should be satisfactory for most clinical epidemiological investigations. This study used visual analysis of autoradiographs, but potentially, a computer linked scanner for radiolabelled proteins would be more convenient and could allow quantitative analysis.

References


Requests for reprints to: Dr JR Stephenson, Department of Medical Microbiology, St Bartholomew’s Hospital Medical College, West Smithfield, London EC1A 7BE, England.