

Rapid inter-strain comparison by pyrolysis mass spectrometry of coagulase-negative staphylococci from persistent CAPD peritonitis

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SUMMARY

Pyrolysis mass spectrometry (PyMS) was used as a method of rapid inter-strain comparison of 19 isolates of coagulase-negative staphylococci from episodes of CAPD peritonitis. Thirteen isolates were from multiple, but distinct, episodes of peritonitis in 6 patients and the remaining 6 isolates were from 6 patients with single episodes. The results, expressed in terms of identity/non-identity of strains, were compared with those obtained using an established typing system comprising an extended antibiogram, determination of biotype and plasmid profile analysis. The PyMS results for inter-strain comparison were in agreement with the reference typing scheme results. PyMS can be used in this setting to rapidly obtain evidence that persistent infection is/is not likely to be due to the same organism, although it cannot be used for formal typing. The results by both methods showed that serial, apparently distinct, episodes of peritonitis over periods as long as 120 days may be due to the same strain of coagulase-negative staphylococcus. Clinically based distinctions between recurrence of infection (same strain) and re-infection (different strains) may not always be supported by the microbiological evidence.

INTRODUCTION

Infection remains the commonest complication of continuous ambulatory peritoneal dialysis (CAPD) [1] and may result in removal of the dialysis catheter. One reason for catheter removal is persistent or relapsing peritonitis which occurs in up to 30% of cases [2].

The commonest organisms encountered in CAPD peritonitis are coagulase-negative staphylococci (CNS), particularly *Staphylococcus epidermidis* [3]. To distinguish between recurrence and re-infection it is necessary to have accurate methods by which different isolates of CNS can be compared. Conventional identification techniques (for instance, API-Staph) will speciate CNS but will not discriminate sufficiently between strains, especially of *S. epidermidis*. Further studies [4] have shown that additional tests such as an extended antibiogram plus

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plasmid profiling and/or phage typing can be helpful in inter-strain comparisons but these latter two techniques are not readily available in many microbiology laboratories.

Typing systems do two things. They characterize individual isolates (typing) and also compare different isolates for evidence of identity/non-identity (inter-strain comparison). It is this second property of the typing system which will distinguish recurrent infection (identical strain) from re-infection (different strains).

A rapid and reliable method for inter-strain comparison alone (that is, without formal type designation) will still permit these clinically important distinctions to be made and so facilitate the investigation of persistent CAPD peritonitis.

Pyrolysis mass spectrometry (PyMS) has recently emerged as a rapid and useful method of inter-strain comparison and promising results were obtained with CNS [5]. Organisms are pyrolysed, the pyrolysates examined by mass spectrometry and the mass spectrograms (serving as transient 'bacterial fingerprints') are analysed and compared mathematically. The results can be presented as dendograms.

We have used PyMS as a rapid method for inter-strain comparison of CNS isolated from multiple episodes of peritonitis in a group of CAPD patients. We evaluated the PyMS results by comparing them with the results of typing the same CNS by a scheme using extended antibiogram + biotype + plasmid profile analysis [4].

MATERIALS AND METHODS

Bacterial strains

Nineteen isolates of CNS from peritoneal dialysis effluent (PDE) samples from patients with CAPD peritonitis were collected over a period of several months from the routine microbiology laboratory at Freeman Hospital. Thirteen strains comprised isolates from multiple but distinct episodes of peritonitis in six patients. Ten of these strains were from two consecutive episodes in five patients, the interval between the two episodes ranging from 17–47 days. The final three CNS from multiple episodes of peritonitis were taken from a single patient experiencing five episodes of peritonitis over a period of 120 days and are the strains isolated from the first, third and fifth of these episodes. The intervals between these isolates were 74 and 46 days respectively.

The remaining six isolates were each taken from single episodes of peritonitis in six separate patients and served as control strains for the methods to be assessed. They also allowed investigation of the possibility that all patients in the unit had become infected with a single strain of CNS as a result of cross-infection. None of the patients had exit site infections during the study period and all episodes of peritonitis were clinically distinct and not examples of relapse during therapy. After isolation each CNS strain had been placed into glycerol broth for storage at -20 °C until required for the study. All subsequent separate techniques (antibiogram, biotype, plasmid profile analysis and pyrolysis mass spectrometry) were performed on single subcultures from this primary source rather than on serial subcultures to minimize the variability of the organisms between the different stages of the investigation.

Antibiogram

After subculture onto nutrient agar (Lab M) and checking for purity, isolates were tested for sensitivity by the comparative disk diffusion method (6) to the following antibiotics: penicillin (5 i.u.), methicillin (10 µg), gentamicin (10 µg), amikacin (30 µg), streptomycin (25 µg), neomycin (30 µg), vancomycin (30 µg), tetracycline (50 µg), erythromycin (5 µg), clindamycin (2 µg), fusidic acid (10 µg), rifampicin (2 µg), trimethoprim (2.5 µg), chloramphenicol (50 µg), novobiocin (5 µg), bacitracin (0.04 i.u.) and furazolidone (100 µg). Sensitivity plates were read after 24 h incubation at 37 °C, except for methicillin sensitivity tests which were incubated at 30 °C. Isolates were recorded as sensitive or resistant, intermediate zone sizes being recorded as resistant.

For bacitracin sensitivity testing, any zone size, however small, was regarded as sensitive.

Biotype

All isolates were examined by the API-Staph method (API Products Ltd, Basingstoke, Hampshire, UK) in a single batch of tests, omitting the lysostaphin test. The strips were inoculated and read after 24 h incubation at 37 °C, according to the manufacturers instructions.

Plasmid profiles

Plasmid profile analysis was performed on the 13 isolates taken from the patients with multiple episodes of peritonitis. Plasmid DNA was extracted following lysis of the cells by lysostaphin and lysozyme (Sigma Ltd) by the technique of Birnboim and Doly (1979) [7] and analysed by electrophoresis after the method described by Meyers and colleagues (1976) [8]. Each distinguishable pattern obtained was assigned an arbitrary number (P1, P2, P3, and so on) to allow comparisons between isolates from the same patient and between patients.

Pyrolysis mass spectrometry (PyMS)

All 19 isolates were subcultured onto single plates of a single batch of nutrient agar (Lab M) and incubated overnight at 37 °C under identical conditions. So that PyMS could be assessed against the conventional techniques each isolate was randomly assigned a number from 1–19 at this stage and PyMS was performed by an investigator unaware of the identity of the strains and of the results of the conventional tests. Each isolate was sampled in triplicate and all 57 samples were processed in a single batch on a Horizon Instruments PYMS 200X pyrolysis mass spectrometer (Horizon Instruments Ltd, Heathfield, Kent, UK).

The apparatus, technique and principles involved have been described previously [9]. Briefly, a well-separated colony of the isolate to be tested was identified and sampled with a flamed straight wire. Care was taken to avoid contamination with the medium. The material was smeared onto a pyrolysis foil (Horizon Instruments) to give a uniform surface coating and the foil was inserted into a pyrolysis tube (Horizon Instruments). Assembled tubes were heated at 80 °C for 5 min in a hot air oven. The time required for the analysis of each sample was approximately 2 min and pyrolysis was at a Curie point of 530 °C for a period

of 4 s. Products were ionised by collision with a crossing beam of low energy (25 eV) electrons, and the ions were separated in a quadropole mass spectrometer which scanned the pyrolysate at intervals of 0·35 s from the start of pyrolysis. Integrated ion counts at unit mass intervals from 11 to 200 were recorded on floppy disk, together with the pyrolysis sequence number and total ion count for each individual sample.

PyMS data analysis

Spectra were normalized by an iterative technique to eliminate variation due to differences in the amount of sample pyrolysed. After normalization replicate spectra of the same strain were labelled as distinct groups and analysed for between-strain variation to select the mass ion peaks showing the greatest ratio of between-strain to within-strain variation. The 30 mass ion peaks showing the greatest discrimination between strains were then subjected to principal component analysis and canonical variate analysis, resulting in a table of Mahalanobis distances [10]. These data were then finally used in a UPGMA analysis to produce a dendrogram [11].

RESULTS

The results of typing of the 19 CNS by antibiogram, biotype and plasmid profile analysis are seen in the Table 1. In patient A the two isolates, which were obtained 24 days apart, are seen to be identical by these methods. This is also true of the two isolates from patient C which were obtained 25 days apart. In patient D, however, the two isolates, which were obtained 23 days apart, are seen to differ in both antibiogram and plasmid profile patterns, despite being of the same biotype of *S. epidermidis*. In patient H all three isolates over a total study period of 120 days are identical by antibiogram, biotype and plasmid profile analysis. This demonstrates that although this patient experienced five distinct episodes of CAPD peritonitis over the 120 day interval, at least three of these episodes spanning the whole period were apparently due to the same strain of *S. epidermidis*. In patient I the differences in antibiogram, biotype and plasmid profiles all point to these two strains, obtained 47 days apart, being totally different CNS. Finally, in patient J the antibiograms and plasmid profiles of the two isolates are identical but the biochemical reactions obtained in the biotype differ slightly, although in both instances the biotype is that of a *S. epidermidis*. These strains were isolated 17 days apart.

Finally, there are sufficient differences in antibiogram and biotypes seen in the CNS from the six single episodes of CAPD peritonitis from unrelated patients on the unit to make it unlikely that any single strain of CNS has been colonizing the patients.

In the PyMS-derived dendrogram (Fig. 1) relationships between the mass spectra of the 19 strains analysed can be seen. On the basis of similarity it can be seen that strains 1 and 16 are more closely related to each other than to any other strain examined and this relationship is also true for strains 13 plus 12. The three isolates 8, 11 and 14 are also seen to be more closely related to each other than to any other strains. Strains 2 and 18, in which the conventional system gave an

Table 1. The results of antibiogram, biotype and plasmid profile testing on 19 isolates of coagulase-negative staphylococci from CAPD peritonitis

| Patient and isolate | Antibiogram* | Biotype and species | Plasmid† profile | PyMS number of strain |
|---------------------|----------------------|------------------------------------|------------------|-----------------------|
| A – episode 1 | b p m g a n tr ch | 6.602.113 <i>S. epidermidis</i> | P1 | 1 |
| A – episode 2 | b p m g a n tr ch | 6.602.113 <i>S. epidermidis</i> | P1 | 16 |
| B – only isolate | b p n t e | 6.606.113 <i>S. epidermidis</i> | ND | 17 |
| C – episode 1 | b f | 6.706.113 <i>S. epidermidis</i> | P2 | 13 |
| C – episode 2 | b f | 6.706.113 <i>S. epidermidis</i> | P2 | 12 |
| D – episode 1 | b p m e c tr | 6.606.113 <i>S. epidermidis</i> | P3 | 3 |
| D – episode 2 | b p m t e tr | 6.606.113 <i>S. epidermidis</i> | P4 | 4 |
| E – only isolate | b p | 6.606.133 <i>S. epidermidis</i> | ND | 10 |
| F – only isolate | b p e | 6.612.052 <i>S. hominis</i> | ND | 15 |
| G – only isolate | b p | 6.606.113 <i>S. epidermidis</i> | ND | 5 |
| H – episode 1 | b p m n | 6.606.113 <i>S. epidermidis</i> | P5 | 8 |
| H – episode 3 | b p m n | 6.606.113 <i>S. epidermidis</i> | P5 | 11 |
| H – episode 5 | b p m n | 6.606.113 <i>S. epidermidis</i> | P5 | 14 |
| I – episode 1 | b p m a g | 6.602.013 <i>S. epidermidis</i> | P6 | 9 |
| I – episode 2 | b p f | 6.606.112 <i>S. epidermidis</i> | P7 | 19 |
| J – episode 1 | b p t tr | 6.614.052 <i>S. hominis</i> | P8 | 18 |
| J – episode 2 | b p t tr | 6.632.050 <i>S. hominis</i> | P8 | 2 |
| K – only isolate | b p m t e tr | 6.430.153 <i>S. simulans</i> | ND | 6 |
| L – only isolate | b p m g a n tr | 6.606.113 <i>S. epidermidis</i> | ND | 7 |

* Antibiogram expressed as drugs to which resistance was demonstrated out of 17 drugs tested (see text) b = bacitracin, p = penicillin, m = methicillin, g = gentamicin, a = amikacin, s = streptomycin, n = neomycin, t = tetracycline, tr = trimethoprim, cl = clindamycin, ch = chloramphenicol, e = erythromycin, f = fusidic acid.

† Plasmid profiles are arbitrarily assigned numbers indicating different patterns obtained. Isolates with the same number, e.g. P1, have identical patterns. ND = not done.

equivocal result, are seen by PyMS to be closely related. Strains 9 and 19 (patient I) and strains 3 and 4 (patient D), however, are seen to be more similar to clinically unrelated isolates than to each other. However, in the latter case (strains 3 and 4) the differences are not large.

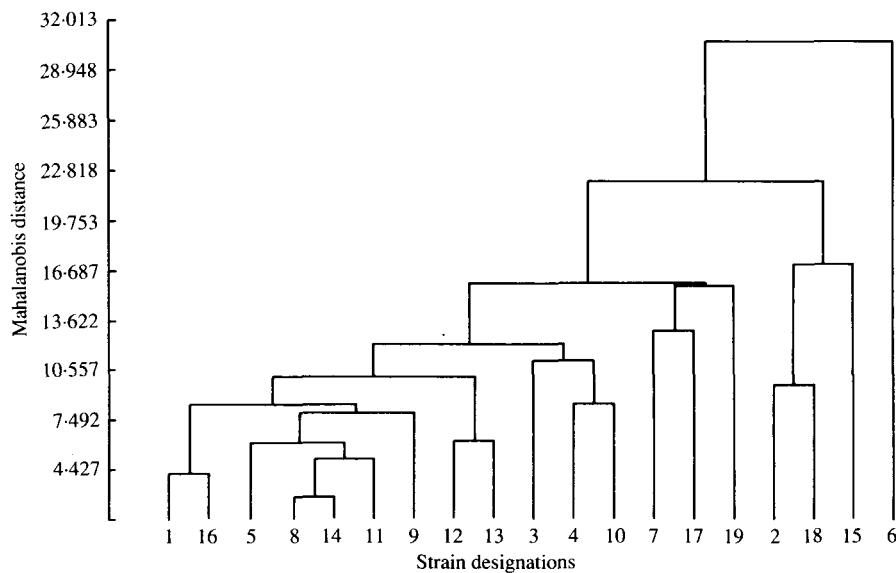


Fig. 1. A dendrogram based on PyMS data. The dissimilarity measure is Mahalanobis distance. The clustering method was UPGMA.

DISCUSSION

Persistent peritonitis is a common problem in CAPD patients [3]. Although relapse during therapy is easily defined, the differentiation between recurrence (defined as the reappearance of symptomatic peritonitis within 2 weeks of completing apparently successful therapy) and re-infection (defined as an apparently new episode of peritonitis beyond this 2-week interval) is much more difficult and these definitions [12] in any case are based on the reappearance of the symptomatic disease, rather than microbiological data. Proof that the same organism was responsible in such difficult circumstances would help clarify this problem.

Microbiological evidence is useful where two isolates are clearly of totally different species or are both of a species within which a well-developed typing system exists, for instance *S. aureus* in which phage typing will be helpful. With the commonest group of organisms involved in CAPD peritonitis, the CNS, speciation is possible using such systems as API-Staph, but unfortunately clinical isolates are almost always the same species, *S. epidermidis*. For this reason attempts have been made to devise typing systems within CNS and especially within *S. epidermidis* [4]. Our study has compared such a scheme with an alternative method of rapid inter-strain comparison (PyMS). The conventional typing tests (antibiogram, biotype and plasmid profile) and PyMS were both shown to be able to provide results of clinical relevance for comparing isolates of CNS from persistent CAPD peritonitis.

The reference typing system we used will result in 76% levels of discrimination between strains [13]. The same study also showed that the biotype component contributed relatively little and could even lead to erroneous results. Plasmid profiling or phage typing were necessary components of the scheme. Both these

latter methods are not commonly available in routine hospital laboratories. Antibiogram alone will give discrimination levels of approximately 50%, which, although acceptable as a primary screening technique, will not serve as a complete scheme. Our alternative strategy using PyMS gave results for inter-strain comparisons which were in complete accord with the reference conventional system. Indeed, PyMS resolved the one situation in which the conventional system gave an equivocal result (patient J).

Inter-strain comparison by PyMS is based on mathematically derived quantitative differences between spectrograms, expressing similarities and differences in relative terms [9]. It is, therefore, essential to include epidemiologically unrelated control strains so that the significance of these relative differences can be estimated. If the differences between spectrograms are not considerable (as between strains 3 and 4), PyMS may require guarded interpretation.

PyMS has been used for inter-strain comparisons of organisms such as salmonellas and *Streptococcus pyogenes* from outbreaks of infective diseases, allowing rapid detection of an epidemic strain [14, 15]. For this reason it may become a more widely available technique in the near future. Apart from the initial capital investment (approximately £80000) it is relatively cheap to run, requires little labour and provides very rapid results. Pyrolysis mass spectrograms are known to vary with the content of the medium and the age of the culture [16], therefore, inter-strain comparisons are only valid when a group of isolates is prepared under identical conditions and examined in a single machine run. Given these conditions, however, the results are highly reproducible.

In the present study PyMS provided results for inter-strain comparison equivalent to the reference system. If PyMS were to be used prospectively for the same purpose, serial isolates from individual patients could be compared within 24 h.

Finally, our results by both methods show that application of such techniques to persistent peritonitis in CAPD patients will indeed be a valuable advance with important clinical implications. We have shown in three patients (A, C and J) that isolates of CNS obtained at intervals of 24, 25 and 17 days, respectively, were identical, whereas in two other patients (D and I) strains obtained at intervals of 23 and 47 days respectively were clearly different organisms. The CNS strains from patient H, who experienced five episodes of CAPD peritonitis over 120 days, were all found to be identical and show that a single strain of CNS can be responsible for multiple episodes of peritonitis over a very long period. The clinically based distinction between recurrence and re-infection [11] may not always be correct. Microbiological examination will add valuable information to the decisions to be taken about catheter removal in such patients.

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