CHARACTERISATION AND TYPING OF BACTERIA

Application of pyrolysis mass spectroscopy and SDS-PAGE in the study of the epidemiology of *Pseudomonas cepacia* in cystic fibrosis

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Summary. Representative isolates of *Pseudomonas cepacia* from 15 cystic fibrosis (CF) patients attending the Respiratory Unit of Alder Hey Children's Hospital were investigated by SDS-PAGE of whole-cell polypeptides and by pyrolysis mass spectroscopy (PMS). SDS-PAGE was less discriminatory than PMS. Eleven isolates were indistinguishable by PMS and considered to represent re-isolates of an endemic strain; four isolates were distinct from this group, and from one another. *P. cepacia* was first isolated on the unit in July 1989 from a patient who had attended a UK selection meeting for a Canadian CF camp. A ward and outpatient segregation policy was introduced, but colonisation of further patients occurred. In August 1991, the Adult CF Association recommended that all social activities involving colonised patients should cease. This, and an increased awareness amongst older CF patients of the risks of person-to-person transmission, was associated with a marked decline in new cases. Social activity and hospital admissions were compared for colonised patients during the year before colonisation with *P. cepacia*, and matched patients who did not acquire the endemic strain. This showed a significantly higher attendance at CF social events for colonised patients, but no significant association between colonisation and hospital admission. These results are strong indirect evidence that transmission of *P. cepacia* occurs through social contact outside the hospital environment.

Introduction

Pseudomonas cepacia has emerged as an important pathogen associated with severe and often fatal infection in children and adults with cystic fibrosis (CF).^{1–3} Acquisition of *P. cepacia* has become a major clinical and psychological problem for CF patients and those involved in their care. Debate concerning the clinical contribution and mode of patient-to-patient transmission with *P. cepacia* led to empirical segregation policies for colonised (Pc+) patients in an attempt to prevent further spread.

Segregation of in-patients, and at out-patient clinics has failed to eliminate cross-infection.⁴ Other workers have shown the importance of reducing non-hospital social contact as means of transmission⁵ and combined phenotypic and genomic typing systems have provided further evidence for transmission of *P. cepacia* by social contact.^{6,7} In the absence of a simple routine typing method, most CF centres, including our own,

have had to adopt segregation with no knowledge of local epidemiology. We report here results of SDS-PAGE and pyrolysis mass spectroscopy (PMS) for isolates from CF patients admitted to the Respiratory Unit of Alder Hey Children's Hospital.

Materials and methods

Patients

All CF patients attended the Respiratory Unit of the Royal Liverpool Children's Hospital, Alder Hey. In May 1991, an in- and out-patient segregation policy for Pc+ CF patients was introduced, and in August 1991, the Adult Cystic Fibrosis Association (ACFA) discontinued its meetings in the Liverpool area in an attempt to reduce social contact between Pc+ and uncolonised (Pc-) patients. Each patient in whom the endemic strain (see *Results*) of *P. cepacia* was identified was matched for age and sex with a Pc- patient. Case and control patients were resident within the Liverpool area. Attendance records of organised events for CF

patients (such as holiday camps and meetings of the ACFA) and hospital admission records were examined for the year before *P. cepacia* was isolated, for patients who were cases and for controls during the corresponding year. Statistical analysis was by the χ^2 test, with Yates's correction.

Isolates

A selective primary isolation medium (Pseudomonas Cepacia Medium; Mast Diagnostic Ltd) for *P. cepacia* was in routine use for a short period in 1989; this was discontinued in 1990 and recommenced in March 1991. Isolates of *P. cepacia* were identified in the API20NE kit incubated at 30°C, and preserved at -70°C. Representative isolates from 15 Pc+ CF patients were examined. Susceptibility patterns were determined in a controlled disk diffusion method⁸ on IsoSensitest Agar (Oxoid) at 37°C in air. Disk contents ($\mu\text{g}/\text{disk}$) were: co-trimoxazole 25, colistin 30, ciprofloxacin 5, gentamicin 10, tobramycin 10, cefoperazone 30, ticarcillin 75, ticarcillin plus clavulanic acid 75+10, imipenem 10, temocillin 30, ceftazidime 30, aztreonam 30, piperacillin 75 and piperacillin plus tazobactam 75+10. *P. aeruginosa* NCTC 10662 was the control organism for susceptibility tests. Colonial morphology and pigment production were scored on IsoSensitest agar after incubation for 48 h.

PMS

Blind-coded cultures of the clinical isolates and of *P. cepacia* NCTC 10661 were subcultured in duplicate on brain heart infusion agar and incubated overnight at 37°C. Well-separated colonies of each subculture were smeared on to pyrolysis foils (Curie point 530°C; Horizon Instruments Ltd); triplicate foils were prepared from each subculture and heated at 80°C for 5 min. All samples were processed as a single batch on a Horizon Instruments PMS 200x pyrolysis mass spectrometer. All six spectra (triplicate samples from each of two subcultures) from each isolate were labelled as a single group.

Spectra were normalised to correct for differences due to variation in sample size. The normalised spectra were analysed in sequential multivariate analyses (principal component followed by canonical variate analysis) to express the statistical inter-group pattern differences in spectra as an ordination diagram on two complex derived axes termed PCCV1 and PCCV2. The ordination diagram was inspected for clusters; spectra for outlying groups were removed and the analysis was repeated until no further outlying groups were identified, as described previously.⁹ This yielded one group of isolates within which the differences between the mean spectral data for each isolate pair were less than the 95% confidence interval for the appropriate number of degrees of freedom (number of groups - 1); i.e., a group of isolates indistinguishable by PMS.

SDS-PAGE

Isolates were coded as for PMS, grown on IsoSensitest agar at 30°C for 48 h and harvested into sterile distilled water. Suspensions were diluted to an absorbance of 2.0 at 570 nm. Equal volumes of bacterial suspension and sample buffer (SDS 4% w/v, sucrose 10% w/v, bromophenol blue 0.025% w/v, 1.4 dithiothreitol 1% w/v in 0.01 M Tris-Tricine-SDS buffer, pH 7.5) were mixed and boiled for 10 min. Samples were clarified by micro-centrifugation at 13000 rpm for 10 min. Electrophoresis was performed in pre-cast 10–20% linear gradient gels (Micrograd, Gradipore Ltd, Pyrmont, Australia) in 0.01 M Tris-Tricine-SDS buffer at pH 7.5 for 3.5 h at 200 V. Protein bands were fixed with trichloroacetic acid 10% w/v and stained with Coomassie Blue (Gradipore Stain, Gradipore Ltd) < 1.0 w/v. Gels were photographed and the relative mobility of proteins was determined by comparison with mol. wt markers (14.2–205 kDa, Sigma).

Results

The most common indistinguishable type as defined by PMS (see below) was considered to be the endemic strain; isolates of this group and the corresponding patients are coded with the prefix E below; non-endemic types are coded N; and isolates that were unavailable for typing are coded U. Isolates N2, N4 and *P. cepacia* NCTC 10661 were non-pigmented. All other strains produced variable levels of brown diffusible pigment and showed a rough colonial morphology. All clinical isolates were resistant to co-trimoxazole, colomycin, ciprofloxacin, gentamicin, tobramycin, cefoperazone, ticarcillin, ticarcillin plus

Fig. 1. Ordination diagram of first spectral data obtained from the examination of 16 isolates of *P. cepacia* by PMS. Individual points represent the mean spectrum data for each isolate (six samples from each isolate) and the circles indicate 95% confidence limits.

clavulanic acid and imipenem (except N2). Susceptibility to temocillin, ceftazidime, aztreonam, piperacillin and piperacillin plus tazobactam was more variable, and the findings have been reported in detail previously.¹⁰ These antibiograms did not differentiate isolates into endemic and non-endemic groups.

PMS

Isolates N1 (PMS code j), N2 (o) and N3 (g) were clearly different from the majority of the isolates and from each other in the first multivariate analysis (fig. 1). Re-analysis after removal of spectra for these strains (fig. 2) resolved two further isolates, *P. cepacia* NCTC 10661 (a) and N4 (m). A third analysis (not shown), after removal of data for these strains, did not differentiate any further outlying strains. The remaining isolates were considered to be indistinguishable by PMS; they comprised E1 (q), E2 (u), E3 (i), E4 (n), E5 (t), E6 (r), E7 (s), E8 (k), E9 (b), E10 (p) and E11 (h).

SDS-PAGE

Typical whole-cell polypeptide profiles are shown in fig. 3. A group comprising clinical isolates N1, N2, N3 and *P. cepacia* NCTC 10661, were resolved from the remainder of the isolates. Profiles were closely similar for most of the clinical isolates; inter-isolate differentiation relied mainly on differences in the three major protein bands characteristically found at 64, 80 and 96 kDa in isolates of the endemic strain. Isolates N1 and N3 produced complementary bands at 70, 84 and 100 kDa, and isolate N2 was clearly distinguished by the absence of a major band in the 80–84-kDa region. Other minor differences in protein profiles could be detected, but were unreliable as differential markers.

Discrimination in PMS and SDS-PAGE was similar; however, PMS differentiated N4 (a non-pigmented strain that gave a typical endemic SDS-PAGE profile), from the endemic cluster, and indicated significant whole-cell composition differences between isolates N1 and N3 that showed similar protein profiles and could not be distinguished reliably in SDS-PAGE.

Epidemiology: spread of the endemic strain

Fig. 4 shows the spread of *P. cepacia* with time, and the introduction of measures to limit hospital and social interaction between Pc+ and Pc- patients. The first isolate of *P. cepacia* in the Alder Hey CF clinic was from patient E1 in July 1989, 1 month after she had attended a UK selection meeting for a Canadian CF camp, but before she attended the Canadian camp. Two further patients (E2, E3) became colonised during the subsequent year. None of these patients had had social contact or concurrent hospital admissions before *P. cepacia* was isolated from their sputum. Representative isolates from these three patients fell within the endemic strain group in PMS and SDS-PAGE.

Fig. 2. Ordination diagram of second spectral data obtained after removal of outliers from the first analysis (i.e., PMS groups g, j and o).

Fig. 3. SDS-PAGE of whole-cell polypeptide profiles of *P. cepacia*. Lanes 1 and 6, mol. wt markers (kDa); 2, *P. cepacia* NCTC 10661; 3, typical endemic strain isolate (E11); 4 and 5, non-endemic strains (N1 and N2).

Primary culture of CF sputa on *P. cepacia* selective agar was discontinued from March 1990 until March 1991. However, the isolation of *P. cepacia* from patient E4 in January 1991 led to the re-introduction of a selective medium in March 1991. During March and

Fig. 4. The epidemiology of *P. cepacia* in the Alder Hey cystic fibrosis clinic.

April 1991, six further patients (E5–E10) were found to have the endemic strain of *P. cepacia*, although the organism had probably been present in their sputum during the preceding year. Patients E4, E8 and E9 had attended a selection weekend for a Canadian CF camp in May 1990. Patients E5 and E7–E10 had been to an “Outward Bound” weekend in September 1990 and had attended meetings of the ACFA during the preceding year. Patient E6 had attended a national CF camp in June 1990. During August 1991 there was one further isolate of the endemic strain from patient E11, who had attended a national CF camp 2 months earlier.

Epidemiology: non-endemic strains

Four patients were colonised with non-endemic strains (fig. 4, N1–N4) and isolates from a further six (U1–U6) await typing. Two of these (U5, U6) acquired *P. cepacia* in 1993, and are not included in fig. 4. Patients N1 and N3 were friends and regularly attended ACFA meetings before initial isolation of *P. cepacia*. The strains appeared similar in SDS-PAGE but were distinguishable by PMS. Patient N2 attended a national CF camp in June 1990 and *P. cepacia* was isolated when the universal use of selective media began (fig. 4). Patient N4 attended a similar camp the following year and a non-endemic strain was isolated from his sputum 2 months later.

Three patients died after acquiring *P. cepacia*, of whom two had the endemic strain. The mean interval between *P. cepacia* acquisition and death was 6 months

(range 2–11 months). Most new isolates of *P. cepacia* occurred after the introduction of the hospital segregation policy. However, after the ACFA ceased meeting in August 1991 there were only two further isolates (one sibling contact and one CF camp). The cessation of these meetings also marked an increased awareness amongst older CF patients of the risk of cross-infection with *P. cepacia*. To investigate the role of social contact in transmission of *P. cepacia* further, a case control study was performed. The mean age of cases and controls was identical (18 years and 3 months). Nine of the 11 patients with the endemic strain of *P. cepacia* had attended at least one organised social event for patients with CF in the year before the organism was isolated. Three of the 11 controls had attended one or more events during the same period. This difference was statistically significant ($\chi^2 = 4.58$, $p = 0.032$). However, when hospital admissions were considered for the same period, there was no significant difference in the number of patients who had one or more admission between cases and controls ($\chi^2 = 0.20$, $p = 0.66$).

Discussion

The need for hospital laboratories to provide typing services for an ever broader range of species has coincided with a period of financial constraint. Consequently, the introduction of ever more sophisticated and discriminatory methodologies, usually involving DNA-based techniques, has been limited to regional

or national centres. Such an approach may be advantageous, with a concentration of expertise allowing better reproducibility and method development. However, there are also advantages to typing by less sophisticated methods at local centres; notably the lack of postal delays, the capability to filter out obviously unconnected isolates from those referred elsewhere, and the direct involvement of local staff. We examined the potential of a relatively simple and inexpensive method, SDS-PAGE, in less specialised centres, to aid in inter-strain differentiation of *P. cepacia* isolates from CF patients. SDS-PAGE was compared with PMS, a method previously found to be comparable in discrimination with a method based on polymerase chain reaction (PCR) amplification of polymorphic regions in the ribosomal RNA genes.¹¹

SDS-PAGE analysis showed that the majority of isolates had similar whole-cell polypeptide profiles. Only three strains were found to be dissimilar, N1, N2 and N3, a finding confirmed by the first analysis of the PMS data. However, the second analysis of PMS data resolved a further strain (N4), and the control collection strain. Furthermore, PMS indicated that isolates N1 and N3, although similar by SDS-PAGE, were distinguishable. SDS-PAGE was capable of inter-strain differentiation only when gross differences occurred in protein profiles and its applicability in the study of person-to-person transmission or environmental acquisition of *P. cepacia* is limited.

The PMS analysis showed the suitability of this approach for rapid inter-strain comparisons of isolates of *P. cepacia*. PMS has been used successfully in the comparative typing of many other micro-organisms,¹² including two further species of the same group, *Xanthomonas maltophilia*¹³ and *P. aeruginosa*.¹⁴ After the initial capital investment, which itself can be amortised, the consumable costs of PMS are <£1/isolate (for two subcultures each pyrolysed in triplicate). The ability to examine many isolates (up to 50/day) by PMS makes it an attractive option for rapid discriminatory investigation of suspected outbreaks, although the validity of comparisons is limited to isolates pyrolysed within a single batch. Nonetheless, for species with no accepted typing scheme, PMS is, at the very least, a rapid and discriminatory method for identifying those isolates upon which more expensive investigations (such as DNA-based comparisons) should be focused.

The results of this study provide strong indirect evidence that transmission of *P. cepacia* occurs predominantly through social contact outside the hospital environment. There was a statistically significant association between attendance of CF social events and colonisation, but no significant association between colonisation and hospital admissions. This is not surprising since in-patients with *P. cepacia* were segregated from the first isolation of *P. cepacia* in the clinic population. However, the peak rate of acquisition coincided with the period when no such segregation was practised at CF social events.

The first documentation of person-to-person transmission of *P. cepacia* was the ribotyping study of LiPuma *et al.*,⁵ who described the transmission of *P. cepacia* between two young adults attending a summer educational programme. They considered that direct person-to-person transmission was the most likely route rather than indirect transmission *via* fomites. However, Nelson *et al.*¹⁵ have shown that colonised patients can contaminate their environment.

Few studies have implicated hospital contact in transmission of *P. cepacia*, either amongst inpatients or outpatients. Burdge *et al.*¹⁶ demonstrated an association between nebuliser use in hospital and acquisition of *P. cepacia* and isolated *P. cepacia* from nebuliser equipment used by patients. However, this was in a type of nebuliser that did not have a disposable water reservoir, unlike the models used in our clinic. Millar-Jones *et al.*¹⁷ observed acquisition of *P. cepacia* in patients who had had a concurrent hospital admission with a known Pc+ patient, but social contact between patients was not documented.

In contrast, several studies have demonstrated the importance of social contact in the transmission of *P. cepacia*. Govan *et al.*⁶ and Smith *et al.*⁷ documented the social contacts associated with outbreaks of *P. cepacia* in Edinburgh and Birmingham, respectively. Govan *et al.*⁶ also showed that social contact was a mode of national transmission between CF centres. As yet, we have not compared our local isolates with those described elsewhere as "epidemic" strains.^{6,7}

Hospital segregation did not prevent the epidemic of *P. cepacia* colonisation that occurred in our clinic. However, the cessation of ACFA meetings on Merseyside, and the accompanying change in social behaviour amongst young adults with CF, was followed by a marked decline in new cases. This finding is similar to those at Edinburgh and Manchester⁶ where no new cases have been reported since June 1992. Moreover Smith *et al.*⁷ suggested that the activities of ACFA in organising national meetings may have inadvertently caused the spread of a single strain of *P. cepacia* between CF centres in the UK.

Clearly if meetings of the ACFA and similar social activities are to be limited, then important social contact and mutual support between young adult CF patients will be lost. An attempt has now been made to define which activities CF patients can safely share, while minimising the risk of *P. cepacia* transmission. The CF Trust *Pseudomonas cepacia* Working Group have recently published a statement on *P. cepacia*¹⁸ suggesting that separate conferences and social activities should be held for Pc+ and Pc- individuals. It also suggests levels of risk of transmission that can be attributed to a number of social activities, from "casual meetings indoors or outdoors", which are low risk, to "sharing eating or drinking utensils" which are high risk. The statement emphasises that adherence to these guidelines does not give complete protection from *P. cepacia* acquisition.

As studies of direct person-to-person transmission

of *P. cepacia* are not feasible, for ethical reasons, it is important that the outcome of implementing these guidelines is closely monitored. Investigation of the

risk factors present in those patients who have recently acquired *P. cepacia* may yield more accurate information on the relative risks of social activities.

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