Pyrolysis typing of isolates from a recurrence of systemic cryptococcosis

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Summary. Cryptococcal meningitis was diagnosed in a 71-year-old male diabetic patient with underlying ischaemic heart disease, asthma and bilateral axillo-femoral vascular grafts. After treatment with fluconazole for 2 months, the patient appeared to be cured. Two years later he presented with an aneurysm of the right graft that was resected and replaced with a new graft segment. Cryptococcus neoformans var. neoformans was grown from post-operative blood cultures and samples of the excised graft. The patient was treated with fluconazole and discharged after 6 weeks. Multiple isolates from both episodes had been preserved, and these, together with isolates from other UK patients, were cultured in duplicate, blind coded and characterised by pyrolysis mass spectrometry (PMS). Duplicate culture and re-isolate sets formed tight clusters, with each patient set clearly distinct. Sets of isolates from the two episodes in this patient formed a single tight cluster and were indistinguishable by PMS. These results support the contention that C. neoformans infection can be reactivated after being dormant for a prolonged period.

Introduction

Cryptococcus neoformans is the causative agent of cryptococcosis. This usually presents as meningitis, although various other, rarer presentations have been described, including the original description of an isolate from a bony lesion with associated cutaneous ulcers. Before the availability of specific treatment with amphotericin B, cryptococcal meningitis was invariably fatal, and the disease still has a high mortality. Many affected patients have some immunological or metabolic defect and cryptococcosis is often regarded as an opportunistic infection. However, a proportion of patients have no such underlying condition, and the reasons why they develop overt disease remain unclear. The incidence of cryptococcal meningitis has increased with the spread of AIDS, and it is now the fourth most common life-threatening infection in HIV-positive patients.

Recent work has shown that either of two varieties of Cryptococcus may be implicated; C. neoformans var. neoformans, and C. neoformans var. gattii. These are the imperfect forms of the basidiomycetes Filobasidiella neoformans var. neoformans and var. bacilliformis respectively, and differ in serology, biochemistry and ecology. C. neoformans var. neoformans is found world-wide in pigeon faeces, is an opportunist pathogen and is now often associated with AIDS. C. neoformans var. gattii is associated with the red river gum tree, Eucalyptus camaldulensis, is confined to Australia and places to which this tree has been exported, is not AIDS-associated and may be a primary pathogen.

An opportunity arose to investigate a series of isolates from a patient who had two episodes of systemic cryptococcosis, separated by an interval of 2 years. Multiple episodes of cryptococcosis are widely believed to be due to reactivation of infection rather than re-infection but there is little direct evidence for this contention. The study was undertaken to find out whether the isolates from the two episodes were indistinguishable, and to demonstrate further the wide-ranging capabilities of pyrolysis mass spectrometry (PMS) as a typing method.

Materials and methods

Patient

Cryptococcal meningitis and cryptococcaemia were diagnosed in patient A, a 71-year-old male with ischaemic heart disease, non-steroid-dependent asthma and insulin-dependent diabetes mellitus. He had bilateral axillo-femoral dacron vascular grafts in situ. Repeated blood and CSF cultures yielded C. neoformans var. neoformans. Cryptococcal antigen was detected in the cerebrospinal fluid (CSF) and blood by latex agglutination (Alpha IMMY kit) at titres of 128 and 32, respectively. He was treated with fluconazole for 6 weeks, and appeared to be cured, with an antigen titre of 2 after 8 weeks.

Two years later he presented with an aneurysm of the right graft, which was excised and replaced with a new graft segment. Blood cultures were taken 2 days...
### Table. Origins of isolates examined

<table>
<thead>
<tr>
<th>PMS blind code</th>
<th>PMS group</th>
<th>Patient</th>
<th>Isolation</th>
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Blind codes for duplicate cultures of the same isolate are indicated on a single line. Isolates for patient A, whose clinical history is described here, are in two sets, those from the initial episode treated at Merthyr Tydfil, and those from the recurrence treated in Cardiff. The location given is the city where the infection was treated.

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**Fig. 1.** A pseudo-three-dimensional ordination diagram representing the PMS typing results. The three axes are complex derived functions that together account for 70% of the statistically significant differences between cultures. Each culture is represented by a point, and the distance between points increases with increasing differences in PMS. Points are labelled with the PMS blind code (see table).
post-operatively because of pyrexia, and these yielded *C. neoformans* var. *neoformans*. The excised graft tissue also yielded this organism after extended incubation. He was again treated with fluconazole, and discharged well after 8 weeks with the intention of maintaining fluconazole treatment indefinitely.

**Cryptococcal strains**

Thirteen isolates were examined (table). These comprised six strains from patient A: three stored isolates from the initial episode and three fresh isolates from the second episode; six stored clinical isolates collected from patients elsewhere in the UK; and a stored isolate identified as *C. laurentii*. Cultures had been stored in sterile distilled water at room temperature and were recovered on Malt Agar (Difco) 3% with gentamicin 0.05% (SMA) incubated aerobically at 30°C for 48 h. The identity of all isolates was confirmed biochemically. All but the isolate of *C. laurentii* gave the following reactions typical of *C. neoformans* var. *neoformans*: brown colonies with round cells and no mycelium on cornmeal agar with caffeic acid (CMCA); no fermentative reactions in sugars; no assimilation of lactose or melibiose; assimilation of glucose, maltose, galactose sucrose, dulcitol, raffinose inositol, trehalose and cellibiose; nitrate reduction negative; urease positive; and no blue colouration on canavanine-glycine-bromothymol blue agar (CGB). The isolate of *C. laurentii* differed in assimilation of lactose and melibiose, failure to produce brown colonies on CMCA, and production of blue colouration on CGB—reactions typical for this species.10

**Pyrolysis mass spectrometry**

Isolates were grown aerobically at 30°C for 72 h on SMA plates poured from the same batch of medium. Stored cultures were serially subcultured at least twice on SMA before analysis; in our experience with other organisms the first subculture from frozen or freeze-dried specimens can give atypical spectra. Isolates were cultured in duplicate or triplicate and all cultures were blind coded. For each subculture, four pyrolysis foils were smeared with colony material, heated to 80°C for 10 min with 10 min of sampling to destroy enzyme activity and dehydrate the specimen, and stored in a vacuum desiccator overnight. The prepared foils were processed in a PYMS 200X automated pyrolysis mass spectrometer (Horizon Instruments, Heathfield, Sussex), pyrolysing for 4 s at 530°C.11

The mass spectra collected were analysed statistically.12 Individual spectra were normalised to correct for variation in sample size, and univariate statistics were calculated. Masses showing within-culture coefficients of variation < 7.5% were submitted to discriminant analysis in the SPSS-PC program suite. This multivariate strategy compares spectra, extracting the significant inter-culture differences as derived axes termed canonical discriminant functions. The sum of the squares of the differences between pairs of spectra on these axes is a measure of the statistical significance of their pattern differences, and, therefore, their compositional differences.13 It approximates to a χ² estimate of significance of difference on (number of cultures − 1) degrees of freedom. Culture-mean coordinates on these axes were then submitted to cluster analysis in the Clustan program suite, with squared Euclidian distance as the dissimilarity measure and UPGMA hierarchical clustering.

**Results**

Univariate analysis of the replicate spectra revealed one culture (blind coded 1) that showed unprecedented variation between replicate spectra; > 30% of the mass intensities in each replicate spectrum of this culture deviated more than expected from the culture mean. The culture plates were re-examined for purity and a small number of slow-growing diphtheroid colonies were found in this aberrant culture. Presumably, the spectrum variation was due to variation in the relative amounts of the cryptococcus and diphtheroid between samples. This re-emphasises the importance of thorough univariate analysis of PMS data13 and of analysis of pure cultures. Spectra of this contaminated culture were discarded from further analysis.

Fourteen cultures gave closely similar spectra, as shown in the ordination diagram and dendrogram

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**Fig. 2.** A dendogram representing the PMS typing results. The level of dissimilarity between cultures is represented by the minimum distance along the axis at which their branch tips become joined in the dendrogram tree. Clear groups are evident, each comprising a set of cultures from a single patient.
(figs. 1 and 2). Five further closely similar culture pairs, one triplet and a single distinct culture were also apparent. One of the culture pairs was a gross outlier. Within these groups, the squared Euclidian distance between culture means was < 48, indicating that cultures were indistinguishable at $p > 0.001$. Squared distances between groups were > 300, indicating significant differences at $p < 0.0001$. When the code was broken, each of the eight groups of strains was found to correspond to a culture set for a single patient. The gross outlier group comprised the duplicate cultures of the C. laurantii isolate (figs. 1 and 2, table). All cultures from the patient described here were indistinguishable, irrespective of the infection episode.

**Discussion**

The unusual presentation of the second episode of disease, survival of both clinical episodes, and, to a lesser extent, the recurrence of infection make this an unusual case. The age of the patient, underlying asthma, heart disease and diabetes seem to have been the predisposing factors in the initial infection. The presentation of the second episode and the PMS typing results strongly suggest that this was a reactivation of the initial infecting organism, rather than a re-infection.

Multiple infection episodes have been described occasionally in systemic cryptococcosis. One case report gives convincing evidence that an infection with *C. neoformans* var. *gattii* lay dormant for at least 8 months and possibly years, before becoming active. like other organisms known to cause reactivating infection, e.g., *Histoplasma capsulatum* and *Mycobacterium tuberculosis*, walled or calcified lesions containing *C. neoformans* have been found in biopsy material or at post-mortem examination of patients showing no evidence of concurrent overt disease. There is also evidence that viable cryptococci may be recovered from the prostate gland of healthy patients after apparently successful antifungal treatment of systemic infection. Localisation of infection at tissue damage sites and grafts is well described for other organisms, particularly for bacterial infections of damaged cardiac valves and valve prostheses. The second episode of infection in this patient may have started as a subclinical pre-operative infection or been triggered by surgery. The possibility of reactivation by trauma or surgery should be borne in mind for patients with a history of overt cryptococcosis. In AIDS, long term prophylactic therapy with fluconazole is now recommended.

PMS proved a rapid, simple and inexpensive typing method for this organism. The only adaptations of the method used routinely for bacterial isolates were changes in culture media and conditions to optimise growth. As in previous studies of PMS typing for other organisms, good inter-strain discrimination was found in a blind trial. PCR, RFLP and electrophoretic karyotyping analyses have been applied in taxonomic studies of cryptococci. These may have some potential application in typing this organism, but none can currently compete with the combination of general applicability, speed, simplicity and economy of PMS.

**References**

18. Vilgalys R, Hester M. Rapid genetic identification and mapping

