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DNA typing of epidemiologically-related isolates of *Aspergillus fumigatus**

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SUMMARY

Invasive aspergillosis is often nosocomially acquired and carries a high mortality. Molecular typing methods to discriminate isolates have now been developed. Using simple restriction endonuclease (*Sal*1 and *Xho*1) digestion of total genomic DNA, we have typed 25 epidemiologically-related isolates of *A. fumigatus* from six hospital episodes of invasive aspergillosis. Eight DNA types were found and in each case the DNA type matched precisely the epidemiological data. Thus DNA typing of *A. fumigatus* can provide the means to match isolates from linked sources and distinguish isolates from diverse origins.

INTRODUCTION

Aspergillus fumigatus is a ubiquitous mould which has been isolated from a wide variety of environments [1]. It is the most common *Aspergillus* species causing human invasive disease. *Aspergillus* species are increasingly important nosocomial pathogens being the causative agents of several disease states including allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis. *A. fumigatus* is a particular threat to immonocompromized patients including transplant patients and those receiving cytotoxic chemotherapy [2]. Recently *Aspergillus* has been identified as an opportunistic pathogen in AIDS patients [3]. In these patient groups the mortality is high.

The occurrence of *A. fumigatus* in the hospital environment is well documented especially where building work is in progress [4–6]. Outbreaks of aspergillosis have

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been associated with potted plants [7], but workers were unable to confirm the exact origin of many of these episodes. This was in part due to the lack of a reproducible, discriminatory typing system for this organism.

Several phenotypic techniques have been applied to distinguish isolates of *A. fumigatus* and these have had limited success [8]. Immunoblotting [9] is probably the most discriminatory phenotypic system that has been used yielding 11 types from 21 isolates. This method was, however, slow, laborious and difficult to standardize. In addition, phenotypic typing systems may be of limited use in assessing relatedness of *A. fumigatus* isolates since strains may possess the same phenotype but have a different genotype.

Recently several DNA based typing methods have been described which can generate useful information for typing *A. fumigatus*. These can be divided into those based on restriction fragment length polymorphisms (RFLP) [8, 10, 11], PCR based systems [12] and those that utilize gene probes [13]. A common feature of most of the typing systems is that they were largely applied to groups of unrelated isolates which would be expected to differ irrespective of the criteria used. DNA typing has yet to be applied to isolates from hospital outbreaks where the same isolate may be present both in the environment and as a disease causing organism. In this study, we have used a RFLP based typing method to examine *A. fumigatus* DNA types from epidemiologically linked hospital isolates.

METHODS

Isolates

Isolates were confirmed as *A. fumigatus* by standard macroscopic and microscopic methods and by growth at 45 °C. Conidia were stored in 10% (v/v) glycerol to maintain optimum viability and subcultured onto Potato Dextrose Agar to produce conidia for inoculation of broths.

The isolates used in this study were AF 200, 201, 202, 203, 204, 205, 208, 209, 210, 211, 212, 213 and 243 from Hope Hospital and AF 245, 246, 247 and 248 from Glasgow, IHEM isolate numbers 5452, 5453, 5454, 5455, 5701, 5702, 5703 and 5711.

Protoplast preparation

Protoplasts were prepared by the method of Denning and colleagues [8] except for the following modifications. Conidia were grown for 20–23 h and mycelia collected by vacuum filtration. Novozyme 234 (InterSpex Products Inc, Foster City, California, USA) and cellulase (Sigma, Poole, Dorset, UK) treatment was for 2–3 h at 33 °C. Deproteination with proteinase K (Sigma, Poole, Dorset, UK) at a concentration of 100 μg/ml was done at 37 °C for 60 min. RNase A (Sigma, Poole, Dorset, UK) was added to the dialysis tubing at a concentration of 10 μg/3 ml. Dialysis was continued for 3 days with 9 changes of buffer. In instances where poor restriction endonuclease digestion was a problem with individual isolates, samples were extracted with 10% (w/v) hexadecyltrimethylammonium bromide (CTAB) in 0·7 M-NaCl prior to phenol:chloroform (1:1) extraction.

Restriction endonuclease analysis

Dialysed DNA solution (265 μl) was transferred using wide bore pipette tips (tips with the ends removed) into a sterile 1·5 ml microfuge tube. Restriction endonuclease digestions (300 μl) contained 50 units of either *Xho*1 or *Sal*1 (Boehringer Mannheim, Lewes, East Sussex, UK) and 30 μl of the manufacturers supplied 10 × buffer. DNA was precipitated by adding 1/2 volume 7·5 M ammonium acetate and 2 volumes 95% (v/v) ethanol followed by incubation at −20 °C for 60 min. After centrifugation at 13000 g for 10 min the pellet was washed in 70% (v/v) ethanol, re-centrifuged as before and air dried for 20 min.

The pellet was resuspended in 20 μl TE (10 mM-Tris-HCL, 1 mM sodium EDTA, pH 8·0) buffer and placed at 37 °C for 2 h to help dissolve high molecular weight DNA. Samples were electrophoresed in 0·4% (w/v) agarose gels at 35 V for 23 h, this regimen helped optimize separation of high molecular weight bands.

Gels were stained with ethidium bromide (0·5 ug/ml) and photographed under UV light using Polaroid 665 film. Black and white enlargements (5″ × 7″) were made from the negatives of the gel images to improve visual discrimination of banding patterns.

In our previous method a typing scheme was developed which scored the number and pattern of bands above and below the heavy bands at 23 kb (*Sal*1) and 21 kb (*Xho*1) down to a constant region at around 12 kb. Using 0·4% (w/v) gels, enhanced separation of high molecular weight fragments was achieved. We now score bands above 18·5 kb following the method of Denning and colleagues [8]. DNA types for episodes 2, 4 and 5 were determined without knowledge of the source of the isolates.

In addition to visual scoring, gel images were analysed using Bio Image Whole Band Analysis (WBA) software (Millipore (UK) Ltd, Watford, UK) on a Sun SPARC Station 2 (Sun Microsystems Inc, Mountain View, California, USA). Images from Polaroid negatives were scanned into the system using a high resolution XRS scanner (Millipore (UK) Ltd, Watford UK) and stored. Bands above the 18·5 kb marker band were automatically identified and assigned by WBA. Lanes were normalized by aligning bright bands at 23 kb (*Sal*1 digests) and 21 kb (*Xho*1 digests). Quantitation of bands was achieved by WBA software, applying the Robust estimation method of Plikaytis and colleagues [14]. Comparison of banding patterns was done automatically, again by WBA software using the match option (using the unweighted pair group method using arithmetic averages, UPGMA).

Computer generated lane maps are shown in Fig. 2

RESULTS

RFLP analysis of *A. fumigatus* genomic DNA using *Sal*1 and *Xho*1 effectively typed the 25 isolates in this study. All RFLP patterns were reproducible between gels and repeat DNA preparations of the same isolate. The majority of detectable RFLPs occurred at high molecular weight so it was essential to use the least destructive techniques in preparing DNA. This was achieved by using wide bore

Table 1. *DNA types of epidemiologically-related* Aspergillus fumigatus *isolates from six hospital episodes*

Isolate No.	Patient episode	Date of isolation	Patient problem, site	DNA type*	
				*Sal*1	*Xho*1
200 AF	1	11.6.91	Liver, neutropenia, corticosteroid therapy	8, 11	10, 8
201 AF	1	11.6.91	Thyroid, autopsy cultures	8, 11	10, 8
202 AF	1	11.6.91	Colon, autopsy cultures	8, 11	10, 8
203 AF	1	11.6.91	Trachea, autopsy cultures	8, 11	10, 8
204 AF	1	11.6.91	Trachea, autopsy cultures	8, 11	10, 8
205 AF	1	11.6.91	Trachea, autopsy cultures	8, 11	10, 8
248 AF	2	23.3.92	BAL, Heart Tx	12, 11	11, 8
245 AF	2	2.6.92	Left eye, Heart Tx	12, 11	11, 8
246 AF	2	12.6.92	Left eye, Heart Tx	12, 11	11, 8
247 AF	2	12.6.92	Left eye, Heart Tx	12, 11	11, 8
208 AF	3	20.5.91	Drain, laparostomy	9, 10	12, 7A
209 AF	3	20.5.91	Liver, laparostomy	9, 10	12, 7A
210 AF	3	21.5.91	Colon, laparostomy	9, 10	12, 7A
211 AF	3	21.5.91	Wound scab, laparostomy	9, 10	12, 7A
212 AF	3	21.5.91	Liver scab, laparostomy	9, 10	12, 7A
213 AF	3	21.5.91	Liver scab, laparostomy	9, 10	12, 7A
IHEM 5452	4	11.90	BAL, I.P.A., Lung Tx	7, 11	12, 7B
IHEM 5455	4	11.90	Outdoor air	7, 11	12, 7B
IHEM 5454	4	11.90	I.C.U. environmental	7, 10	13, 8
IHEM 5453	4	11.90	I.C.U. environmental	7, 12	8, 8
IHEM 5701	5	23.3.91	Freeze dried synthetic dura mater, neurosurgery	9, 11	11, 6
IHEM 5702	5	8.4.91	Skin surgical wound, neurosurgery	9, 11	11, 6
IHEM 5703	5	11.4.91	Clot under dura mater, neurosurgery	9, 11	11, 6
IHEM 5711	5	11.4.91	Artificial dura mater, neurosurgery	9, 11	11, 6
243 AF	6	16.3.92	Mastoids, external ear	7, 12	11, 7

IHEM, Institute of Hygiene and Epidemiology; BAL, Bronchoalveolar lavage; I.P.A., Invasive pulmonary aspergillosis; I.C.U., Intensive care unit; Tx, Transplant.

* Reference 8.

pipettes at all times to reduce shear forces and by keeping sample transfer to a minimum.

The protoplast lysate method produced high molecular weight DNA of between 60 and 100 kb but at low concentration. This problem was overcome by using large volume restriction digests followed by a DNA precipitation step after endonuclease digestion. Optimal fragment separation was achieved by using low percentage agarose gels and extended electrophoresis times. The 0·4% (w/v) agarose gels provides better discrimination between isolates than the 0·65–0·7% (w/v) gels used previously [8, 11]. Computer aided analysis of banding patterns was in total agreement with visual comparisons. After alignment of bright bands, isolates identified as having indistinguishable banding patterns by visual comparison were matched to a level > 99% similarity by WBA.

The six patients episodes covered a variety of clinical manifestations of

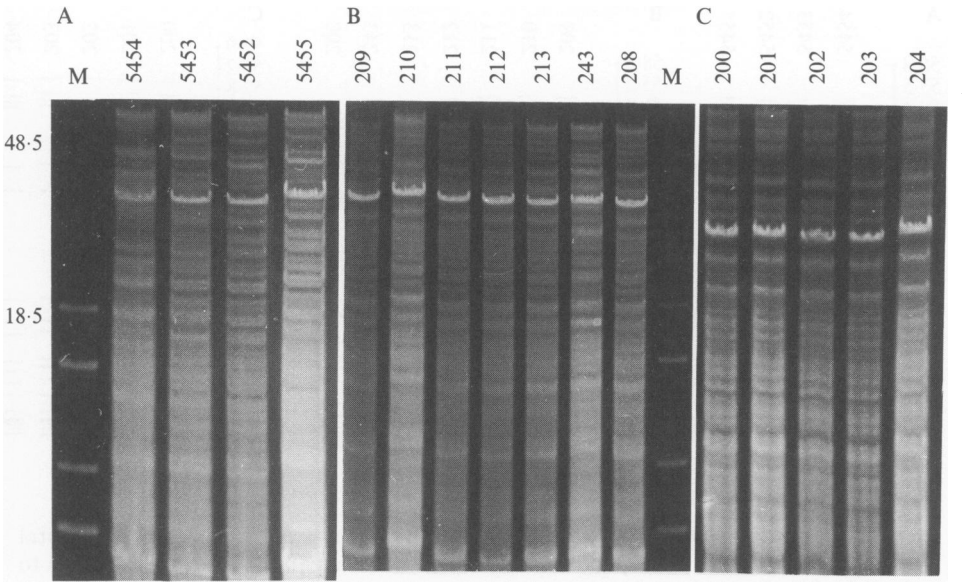

Fig. 1. Restriction endonuclease generated DNA fragment profiles of *A. fumigatus* total cellular DNA. Panel A, *Sal*1 digest of isolates from patient episode 4; Panel B, *Sal*1 digest of isolates from patient episodes 3 and 6; Panel c, *Xho*1 digest of isolates form patient episode 1. Isolates labelled as Table 1. M; molecular weight markers (kb).

aspergillosis. In episode one a neutropenic patient on corticosteroid therapy developed pseudomembraneous aspergillus tracheobronchitis [15]. Autopsy cultures of several sites grew *A. fumigatus*. The DNA types of the six isolates (see Table 1, Fig. 1, Panel C) were indistinguishable, consistent with clonal dissemination of one isolate throughout the body.

Bronchoalveolar lavage from patient two was positive for *A. fumigatus* some 3 months before the organism was isolated from the left eye. Again the DNA types of all the isolates were indistinguishable. This indicates a relatively long incubation time before the development of invasive aspergillosis in this patient.

In patient three a laparostomy was fashioned after multiple abdominal operations and wound dehiscence. After 48 h dark patches were seen on the liver and colon and biopsy from the liver confirmed invasive aspergillosis. Culture of each of the dark areas yielded *A. fumigatus*, all of which had an indistinguishable DNA type (Fig. 1, Panel B). No environmental isolates were found despite the use of 30 settle plates in the vicinity of the patient. Patient 6 was on the same ward as patient 3 some 10 months later: *A. fumigatus* was the only organism isolated from an external ear infection and was of a different DNA type than patient 3 (Fig. 1, Panel B).

In episode four the DNA type from a patient with invasive pulmonary aspergillosis was the same as an outdoor air isolate but distinguishable from two ICU environmental isolates in which the patient was cared for (Fig. 1, Panel A). The implication in this case is that the infecting isolate was not nosocomially acquired but acquired prior to admission.

Patient five developed invasive aspergillosis after receiving synthetic dura mater during neurosurgery. Subsequent analysis of the artificial dura mater

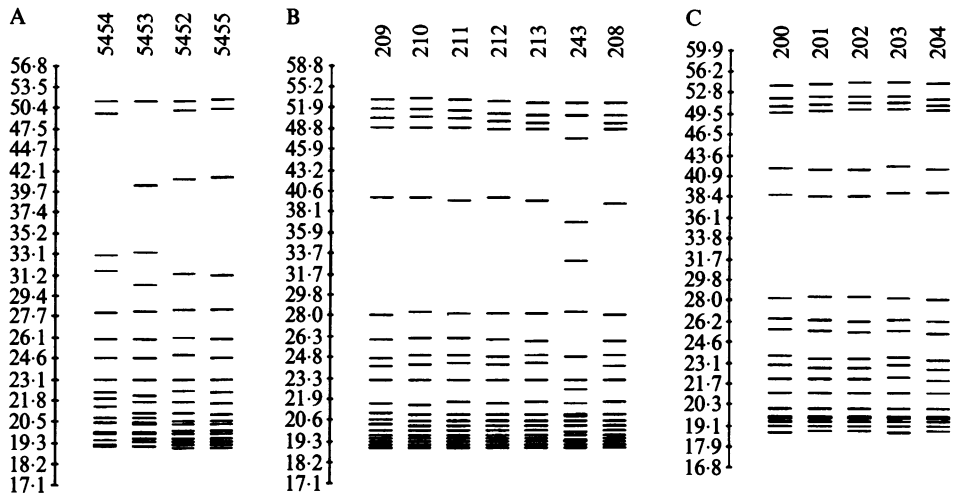

Fig. 2. Computer generated lane maps of DNA fragment profiles of *A. fumigatus* total cellular DNA. Panels correspond to Fig. 1. Molecular weight markers (kb) are given to the left of each panel.

confirmed contamination with *A. fumigatus*. DNA typing confirmed all isolates from this episode to be the same.

DISCUSSION

Twenty-five isolates of *Aspergillus fumigatus* from six different patient episodes revealed eight different DNA types (see Table 1). In four patient episodes one DNA type only was identified whilst in one episode three DNA types were found. No DNA type was shared amongst the six different patient episodes.

Previous RFLP analysis of *A. fumigatus* DNA following *EcoR*1 digestion yielded poor differentiation between isolates [8, 10, 13]. Burnie and colleagues [10] also used *Xba*1 to delineate six types from 21 isolates based on the variation in position of bright bands between 1 and 16 kb. Whilst this method has been applied to other fungi it is not applicable to the high molecular weight polymorphisms exhibited in our study using *Sal*1 and *Xho*1. Here only one bright band occurs and differentiation is based on the pattern of bands above and below it. We have found that our typing scheme adequately differentiates isolates and is highly reproducible.

Other genotypic methods used to fingerprint *A. fumigatus* includes the random amplification of polymorphic DNA [16]. This technique uses short oligonucleotides of arbitrary sequence to amplify unknown regions of a genome. Depending on the primer used a pattern of bands can be generated specific for the species or strain. This technique has been applied to *A. fumigatus* [12] with encouraging results. The procedure does not require high quality DNA and is rapid. Optimization for each thermal cycler is required so comparison of results generated with different machines may prove difficult.

Many different typing systems have been developed for *A. fumigatus*. One recent phenotypic method developed by one of the authors, using polyclonal and

monoclonal antisera, yielded identical results with the same isolates from episodes 4 and 5 in this study [18]. The discriminatory power of the various techniques needs to be rigorously compared. It is clear that multiple clones (DNA types) exist but the full epidemiology of invasive aspergillosis has yet to be worked out.

In this study, no patient was infected with more than one isolate although this has been occasionally demonstrated in respiratory secretions previously [8, 10, 11]. In patient one, studied at autopsy, indistinguishable RFLP patterns in all six isolates suggests disseminated infection although we cannot absolutely exclude cross contamination at autopsy. The long incubation period observed in patient 2 between BAL positivity and the ocular infection is of interest and not previously documented with powerful typing methodology. If true for other patients, prophylaxis or pre-emptive therapy might prevent the development of disease.

The finding that all six isolates from patient 3 were indistinguishable and appeared within 48 h of each other suggests a point source for infection. Despite the absence of environmental isolates, the common DNA type suggests a 'signature' type for this intensive care unit. This could be confirmed by analysing more isolates, both clinical and environmental, from the unit. Signature types were also seen in the previous study from Stanford University Hospital [8]. In the case of episode 4 two DNA types were found in the intensive care unit indicating that longitudinal studies in hospital settings are required to determine one or more signature types. Full delineation of a hospital outbreak is thus likely to require DNA typing of multiple isolates and more sensitive means of culturing than settle plates, such as air sampling.

The positive culture from the environment of *A. fumigatus* during an outbreak of aspergillosis is potentially helpful as it may now be possible to accurately delineate the source of the isolate e.g. episode 4. This is important in identifying sources of contamination and may allow directed preventative measures to be implemented. It may also have medicolegal implications if it is unclear whether patients entered the hospital harbouring the isolate or acquired it in the hospital, eg. episode 6. Furthermore, perioperative or postoperative infections may now be traceable epidemiologically e.g. episodes 3 and 5. This would allow infections due to airborne spores or contaminated surgical materials to be distinguished. However, isolates from operating room air have yet to be DNA typed.

This study adds substantially to the body of knowledge concerning the epidemiology of nosocomial aspergillosis. Meaningful data can be generated on the likely source of a given infecting isolate from hospitalised patients. Prevention strategies can be rigorously assessed.

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