J Clin Pathol 1992;45:324-327

Restriction endonuclease analysis of *Aspergillus fumigatus* DNA

J P Burnie, A Coke, R C Matthews

Abstract

Aims: **To develop a genome based DNA fingerprinting system for *Aspergillus fumigatus* mould.**

***Methods*:** **DNA was extracted from 21 isolates obtained from eight patients with an aspergilloma. This was with a freeze-dried mycelial extract fragmented in liquid nitrogen. DNA was subsequently purified by phenol-chloroform extraction followed by ultracentrifugation on a caesium chloride gradient. The DNA was restricted by *EcoRI* and *Xba I*.**

***Results*:** **All isolates were identical when cut by *EcoRI*; *Xba I* delineated six DNA types.**

***Conclusions*:** **DNA fingerprinting can be used to type isolates of *A fumigatus*. Strains from within an aspergilloma which were morphologically distinct could either have the identical DNA fingerprint or produce a unique type.**

Aspergillus fumigatus has recently been described as a cause of nosocomial infection producing clusters of invasive disease that was associated with hospital building work.[1-3] It is important to be able to differentiate isolates as outbreaks due to a single source such as an infected air filter can be distinguished from a cluster of cases due to an overall increase in the spore count.

Conventional techniques such as phage typing, antibiograms, and plasmid profiles are clearly inappropriate with fungal moulds. Immunoblot fingerprinting has been previously applied to *A fumigatus* and this phenotype based system generated 11 types among the 21 isolates from eight patients with an aspergilloma.[4] This technique was laborious and involved growing up each isolate and fragmenting it by mechanical pressure. The resulting supernatants were run on a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transblotted on to a nitrocellulose membrane. They were developed in a modified enzyme linked immunosorbent assay (ELISA) with a rabbit hyperimmune antiserum raised against *A fumigatus* NCPF 2109.

In recent years there has been an upsurge of interest in genotype based restriction fragment length polymorphisms (RFLPs) of total cellular DNA as a means of typing microorganisms. This technique has been successfully applied to several human fungal pathogens including *Candida albicans*,[5,6] *Torulopsis glabrata*,[7] *Candida parapsilosis*,[8] *Candida tropicalis*,[9] *Histoplasma capsulatum*,[10] *Trichophython rubrum*[11] and *Absidia glauca*.[12] Distinction between isolates depended on differences in either mitochondrial or ribosomal DNA.[13] In the case of *C albicans* 16 different genotypes were identified among 45 isolates from five outbreaks of systemic candidosis and 94 control isolates. This was with the restriction enzyme *EcoR I*.[5]

Department of Medical Microbiology, University of Manchester Medical School, Oxford Road, Manchester M13 9PT
J P Burnie
A Coke
R C Matthews

Correspondence to:
Dr J P Burnie.

Accepted for publication
23 September 1991

Methods

Twenty one isolates from eight cases of aspergilloma were examined. They were identified as *A fumigatus* by their standard cultural characteristics and microscopic appearance. *A fumigatus* NCPF 2109 was the control isolate.

Each isolate was subcultured on to Sabouraud's dextrose agar and grown at 30°C for 48 hours. It was inoculated into Sabouraud's dextrose broth and grown for a period of three to five days at 30°C in an orbital shaker rotating at 3000 rpm. The mycelium was harvested by filtration and the solid culture was freeze-dried overnight at 4°C. The freeze-dried mycelium was harvested in liquid nitrogen. To each gram of white suspension 10 ml of extraction buffer was added. Each 100 ml contained 20 ml 1 M TRIS pH 8, 25 ml 1 M sodium chloride, 5 ml of 0·5 M EDTA, pH 8·5, 2·5 ml 20% SDS and 47·5 ml of sterile distilled water. This was mixed with a bulb pipette and stored on ice for 30–60 minutes. It was split in two aliquots of 5 ml. To each was added 3·5 ml of phenol and 1·5 ml chloroform:isoamyl alcohol. It was spun for one hour at 5000 rpm. This was repeated and 200 µl of 20 mg/ml of RNase was added to each preparation. This was incubated in a water bath at 37°C for 30 minutes. The nucleic acid was phenol/chloroform and chloroform extracted. It was precipitated overnight at −20°C in ethanol. It was spun at 4°C for 20 minutes at 10 000 rpm.

After centrifugation the precipitated nucleic acid was washed with 70% cold ethanol. The precipitate was dried and resuspended in 3 ml TE of (10 mM TRIS-hydrochloride buffer, pH 7·5, 1 mM EDTA, pH 7·5).

To every ml of sample 1 g of CsCl was added. The volume was made up to 10 ml in a Beckman tube. Ethidium bromide (300 µl of 10 mg/ml) was present in each tube. Samples were centrifuged at 48 000 rpm at 17°C for 70 hours. The DNA band was collected after centrifugation.

The ethidium bromide was removed by

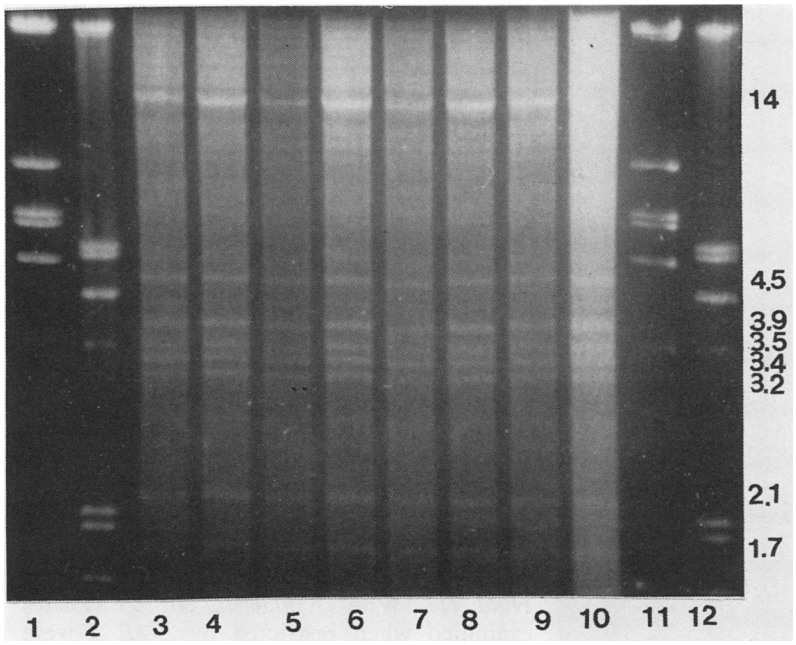

Figure 1 Tracks 1, 2, 11 and 12 molecular weight markers. Tracks 3–10 EcoR I digested A fumigatus isolates.

mixing with equal volumes of 1-butanol saturated with distilled water. The aqueous phase was removed and the extraction repeated six times until the pink colour was removed from both phases. This was checked under ultraviolet light. The CsCl was removed by diluting the DNA with twice the volume of distilled water. The DNA was precipitated by two volumes of ice cold absolute ethanol at −20°C overnight. It was centrifuged at 10 000 rpm for 15 minutes at 4°C. It was washed in 70% ethanol (2×). The DNA was dried at 55°C for 10 minutes. Sterile distilled water (360 μl) was added to dissolve the DNA at 55°C for 20 minutes. TE buffer (40 μl of 10×) was then added.

Reaction buffer (10 μl) and 5 μl of enzyme (NBL Enzymes Division, Cramlington) (*EcoR I* or *Xba I*) and 45 μl distilled water were added to the DNA, giving a final volume of 100 μl. Endonuclease digestion was carried out to completion by incubating at 37°C for two hours. The digestion was stopped by heating the mixture to 70°C for 10 minutes.

Twenty per cent Ficoll (5 μl) was added to the digested DNA sample and also to both the *Hind III* digested λ DNA and *Hind III*/*EcoR I* digested λ DNA (150 mg/ml) as size markers. Electrophoresis of these samples was carried out overnight at 40 volts (constant voltage) in a 300 ml horizontal gel containing 0·8% agarose and 15 μl of ethidium bromide (10 mg/ml). The gel was run in TRIS-borate-EDTA buffer.[14] After electrophoresis the bands were visualised under ultraviolet light and photographed with a Polaroid type 57 film.

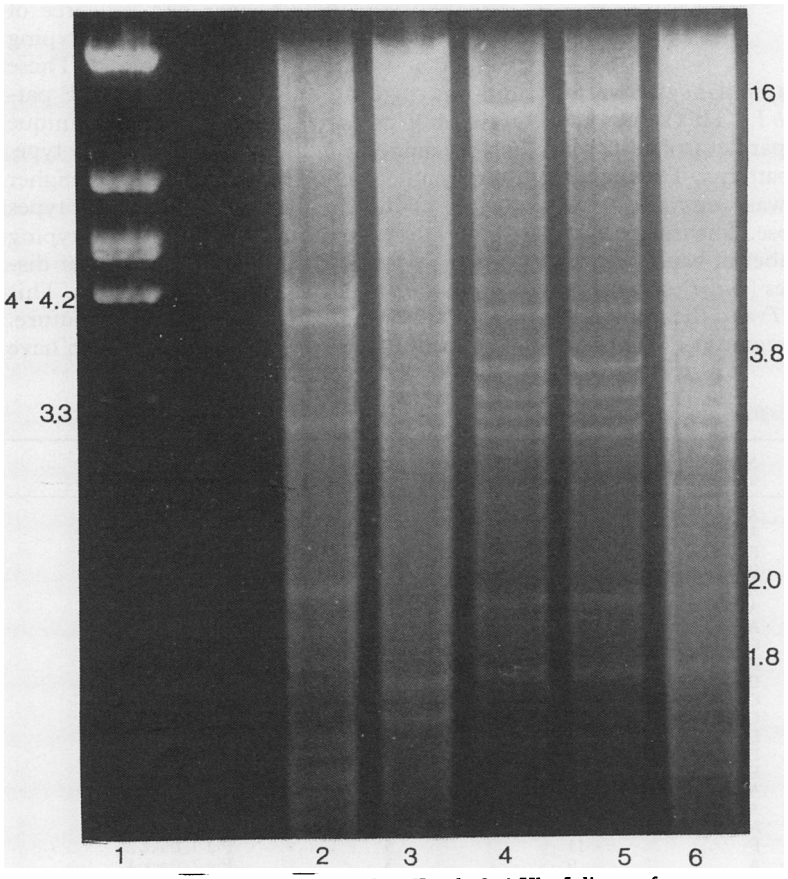

Figure 2 Track 1 molecular weight marker. Tracks 2–6 Xba I digests of A fumigatus isolates 2, 18, 20, 21 and 15, respectively.

Results

Typing systems can be assessed by three criteria: typability, reproducibility, and discrimination. All isolates were typable with both enzymes and reproducibility was excellent between gels. Each isolate was examined at least three times. The enzyme *EcoR I* produced no discrimination between isolates. Bright bands were detected at 14, 4·5, 3·9, 3·5, 3·4, 3·2, 2·1 and 1·7 kilobases. Eight of the isolates are illustrated in fig 1. These represent a single isolate from each case and the control isolate *A fumigatus* NCPF 2109 in track 3.

Xba I delineated six DNA types. It showed bright variable bands at 16, 4–4·2, 3·8, 3·3, 2 and 1 kilobases. The criteria for distinguishing between isolates are summarised in table 1. DNA types 1, 2, and 3 are illustrated in fig 2 and DNA types 4, 5, and 6 in fig 3. The different DNA types described were correlated with the results previously obtained by immunoblot fingerprinting in table 2.[4] *A fumigatus* NCPF 2109 produced an identical DNA type to the isolate from case 1. In cases 1 and 2 only a single isolate was available for examination. In cases 5 and 6 variation in immunoblot fingerprints did not correlate with differences in RFLP types. In cases 3, 4, and 7

Table 1 Details of six types of A fumigatus generated by DNA fingerprinting

Band (kilobases)	DNA types (Xba I digest)					
	1	*2*	*3*	*4*	*5*	*6*
16	−[2]	−	+	−	+	−
4–4·2	Triple band	+[3]	+	+	−	+
3·8	−	−	+	+	−	−
3·3	+	Double band	Triple band	+	Trace	+
2·0	+[1]	−	+	−	−	−
1·8	+[1]	+	+	−	−	−

[1] = band is high; [2] = band at 8·6 kilobases; [3] = band is low.

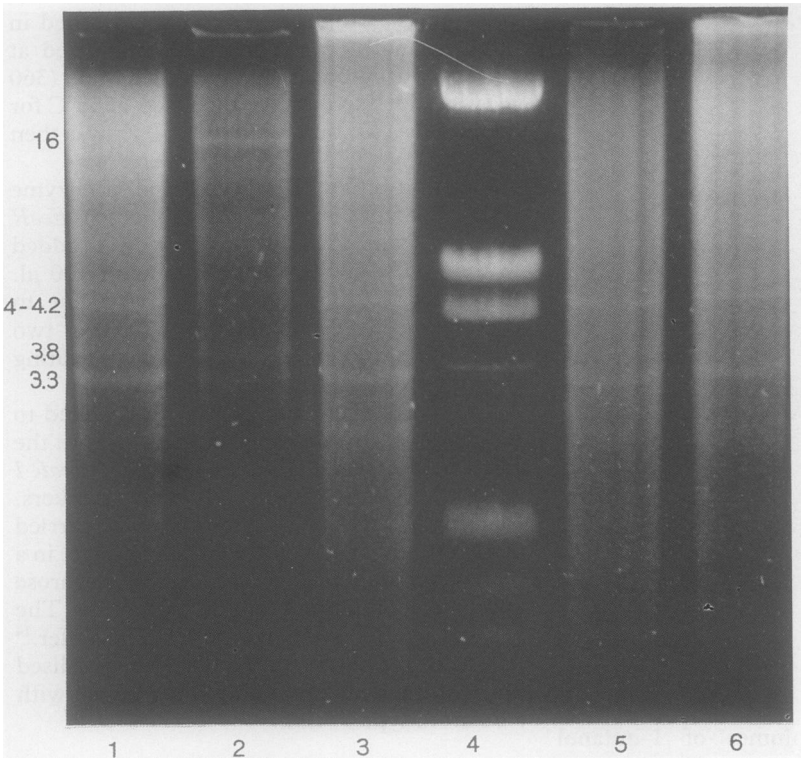

Figure 3 Track 4 molecular weight marker. Tracks 1–3, 5 and 6 Xba I digests of A fumigatus isolates 1, 19, 8, 11 and 13, respectively.

the multiple isolates from within the same aspergilloma produced identical immunoblot and RFLP types. In case 8 variation in immunoblot profile correlated with the presence of three separate RFLP types from within the lesion.

Discussion

Denning *et al* developed a similar system using the enzymes *Sal I* and *Xho I*.[15] This subdivided 31 epidemiologically separate isolates from three continents into 24 patterns. The method of DNA extraction was enzymic with novozyme 234 and cellulose. The isolates were differentiated by the number of bands above a heavy band at 23 kilobases (*Sal I*) or between 21 and 23 kilobases (*Xho I*) and the number of bands down to a constant region at 12 kilobases. These criteria differ from most typing systems involving restriction fragment length polymorphisms where isolates are distinguished by variation in the brighter DNA bands. The use of faint bands is questionable as these are the least likely to be reproducible. They were used because the yield of DNA following enzymic extraction was low.[15] This problem was encountered by ourselves and overcome by the technique presented here involving the extraction of DNA from a freeze-dried extract.[16]

RFLP analysis has been used successfully to type both *C albicans*[5,6] and *Candida tropicalis*.[9] In both cases variation in the position of bright DNA bands was used to delineate between strains and identify cross-infection. The application of the technique to *Candida parapsilosis*[8] and *Torulopsis glabrata*[7] was less rewarding. With *C parapsilosis* all isolates were identical when digested with the enzymes *EcoR I, Bam HI, Kpn I, Bgl II, Hpa II, Pvu II* or *Hind III*. With *T glabrata* all 33 isolates examined when restricted by *EcoR I* were indistinguishable. *Xba I* delineated five types of which one was responsible for an outbreak.

In the case of *A fumigatus* all 21 isolates from the eight patients with an aspergilloma were identical when restricted with *EcoR I. Xba I* identified six types (table 2). Denning *et al* showed that patients harboured multiple isolates of *A fumigatus* in their pulmonary secretions concurrently and over time.[15] The results presented here show that aspergillomas may contain isolates of the same genotype (cases 3, 4, 5, 6, 7) or different genotype (case 8). *A fumigatus* has been previously typed by both silver staining SDS-PAGE and immunoblotting.[4] The former had a degree of discrimination similar to the RFLP typing method and defined six types (table 2). These did not correlate exactly with the RFLP patterns, but in case 8, isolate 18 had a unique silver stain, immunoblot, and RFLP type. Immunoblot fingerprinting had a higher degree of discrimination producing 11 types (table 2). The combination of the three typing systems permitted an adequate degree of discrimination between *A fumigatus* isolates. This will be applied epidemiologically in the future. The results show that the isolates, which have

Table 2 Details of RFLP types from aspergilloma isolates

No of isolates	*Case No*	*Silver stain type*	*Immunoblot type*	*RFLP type*	*Illustration*
1	1	A	1	4	Fig 3, track 1
2	2	A	2	1	Fig 2, track 2
3	3	B	3	3	
4	3	B	3	3	
5	4	A	4	3	
6	4	A	4	3	
7	4	A	4	3	
8	5	C	5	6	Fig 3, track 3
9	5	D	6	6	
10	5	D	6	6	
11	6	C	7	6	Fig 3, track 5
12	6	C	7	6	
13	6	D	6	6	Fig 3, track 6
14	7	E	8	3	
15	7	E	8	3	Fig 2, track 6
16	8	A	9	3	
17	8	A	9	3	
18	8	A	10	2	Fig 2, track 3
19	8	F	11	5	Fig 3, track 2
20	8	A	9	3	Fig 2, track 4
21	8	A	9	3	Fig 2, track 5

been shown to be morphologically distinct[13] from within an aspergilloma can be differentiated both in terms of pheno- and genotype.

RCM is a Wellcome Senior Research Fellow. The award was supported by a grant from The Trustees of the Manchester Medical School.

1 Opal SM, Asp AA, Cannady PB, Morse PL, Burton LD, Hammer PG. Efficacy of infection control measures during a nosocomial outbreak of disseminated aspergillosis associated with a hospital construction. *J Infect Dis* 1986;**153**:634–7.
2 Perraud M, Piens MA, Nicoloyannis N, Girard P, Sepetjan M, Garin JP. Invasive nosocomial pulmonary aspergillosis: risk factors and hospital building works. *Epidemiol Infect* 1987;**99**:1147–50.
3 Dewhurst AG, Cooper MJ, Khan SM, Pallett AP, Dathan JRE. Invasive aspergillosis in immunosuppressed patients: potential hazard of hospital building work. *BMJ* 1990;**301**:802–4.
4 Burnie JP, Matthews RC, Clark I. Milne LJR. Immunoblot fingerprinting Aspergillus fumigatus. *J Immunol Methods* 1989;**118**:179–86.
5 Matthews RC, Burnie JP. Assessment of DNA fingerprinting for rapid identification of outbreaks of systemic candidiasis. *BMJ* 1989;**298**:354–7.
6 Scherer S, Stevens DA. Application of DNA typing methods to epidemiology and taxonomy of Candida species. *J Clin Microbiol* 1987;**25**:675–9.
7 Lee W, Burnie JP, Matthews RC, Oppenheim BO, Damani NN. Hospital outbreaks with yeasts. *J Hosp Infect* 1991;**18** (Supplement A) 237–49.
8 Carruba G, Pontieri E, de Bernardis F, Martino P, Cassone A. DNA fingerprinting and electophoretic karyotype of environmental and clinical isolates of Candida parapsilosis. *J Clin Microbiol* 1991;**29**:916–22.
9 Doebbeling BN, Hollis RJ, Isenberg HD, Wenzel RP, Pfaller MA. Restriction fragment analysis of a *Candida tropicalis* outbreak of sternal wound infections. *J Clin Microbiol* 1991;**29**:1268–70.
10 Vincent RD, Goewert R, Goldman WE, Kobayashi GS, Lambowitz AM, Medoff G. Classification of Histoplasma capsulatum isolates by restriction fragment polymorphisms. *J Bacteriol* 1985;**165**:813–16.
11 de Bievre C, Dauguet C, Nguyen VH, Ibrahim-Granet O. Polymorphism in mitochrondrial DNA of several Trichophyton rubrum isolates from clinical specimens. *Ann Inst Pasteur Microbiol* 1987;**138**:719–27.
12 Wostemeyer J. Stain-dependent variation in ribosomal DNA arrangement in Absidia glauca. *Eur J Biochem* 1985;**146**:443–8.
13 Leslie CE, Flannigan B, Milne LJR. Morphological studies on clinical isolates of Aspergillus fumigatus. *J Med Vet Mycol* 1988;**26**:335–41.
14 Maniatis T, Fritsch EF, Sambrook J. *Molecular cloning: a laboratory manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1978.
15 Denning DW, Clemons KV, Hanson LH, Stevens DA. Restriction endonuclease analysis of total cellular DNA of Aspergillus fumigatus isolates of geographically and epidemiologically diverse origin. *J Infect Dis* 1990; **162**:1151–8.
16 Specht CA, DiRusso CC, Novotny CP, Ullrich RC. A method for extracting high-molecular-weight deoxyribonucleic acid from fungi. *Analyt Biochem* 1982; **119**:158–63.